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# The post-translational methylation of arginine in the glycine arginine rich region of CHO nucleoin

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THE POST-TRANSLATIONAL METHYLATION OF ARGININE IN THE GLYCINE  
ARGININE RICH REGION OF CHO NUCLEOLIN

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

in

The Department of Biological Sciences

by  
Gregory James Pellar  
B.S. University of Mississippi, 1994  
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## **Dedication**

This dissertation is dedicated to my parents, Ernest J. Pellar and Francis C. Pellar who raised me with freedom of choice, and gave me boundless possibilities. I could not have reached this point without their love and support.

## **Acknowledgments**

I would like to thank all those who helped me during my graduate education. First I would like to thank my graduate committee, Dr. Sue Bartlett, Dr. Grover Waldrop, and Dr. Ding Shih, for their time and effort on my behalf. My graduate advisor, Dr. Patrick DiMario, who taught me patient determination, even in the face of impossible odds and did not give up on me, even when I felt like giving up.

I was surprised by the open and sharing nature of the academic science community. I am thankful to Michael Henry and Pam Silver (Harvard Medical School, Dana-Farber Cancer Institute, Boston, MA) for providing me with the Hmt1p cDNA. I thank Dr. Benigno Valdez (Baylor College of Medicine, Houston, TX) for providing our laboratory with antibodies directed against human fibrillarin and B23. I would also like to thank Tom Meier (Albert Einstein College of Medicine of Yeshiva University, NY) for providing antibodies against human Nopp140.

I would like to thank fellow graduate students Matt Brown, and Anwar Khan for reminding me that science is as much a dynamic discussion as it is working at a bench. I would especially like to thank Anwar Khan and Dr. Shih's lab for use of their equipment and materials, without which I could not have completed my work. Finally, I would like to thank my wife, Leah, who has dealt with my testing her patience and who helped me in the final push to produce this dissertation.

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## Abstract

Nucleolin is a nucleolar protein important for ribosome biogenesis. Nucleolin contains a conserved glycine arginine rich (GAR) domain near its carboxy terminus. GAR domains are defined by repeating tri-peptide, (arginine -glycine-glycine (RGG)) motifs. The arginine in the RGG motif is post-translationally modified by dimethylation on one of the two guanido nitrogens. Although arginine methylation was identified over 30 years ago, the function of this modification remains unknown. The GAR domain of nucleolin is important for nucleic acid binding and for nucleolar localization of nucleolin. This dissertation describes investigates possible *in vivo* interactions between nucleolin and other nucleolar proteins involved in ribosome biogenesis. This dissertation also addresses the possible roles that arginine dimethylation may play in the function of the GAR domain.

A GAR truncated hamster nucleolin ( $\Delta$ GAR) localizes to the nucleoplasm and not to the nucleoli of CHO cells. This version of nucleolin was subsequently used to probe the *in vivo* interactions of nucleolin with other non-ribosomal nucleolar proteins. In support of previous work,  $\Delta$ GAR caused endogenous B23 to redistribute to the nucleoplasm, suggesting an *in vivo* interaction. Endogenous Nopp140 shows no redistribution in the presence of  $\Delta$ GAR. The effect of  $\Delta$ GAR on the nucleolar protein, fibrillarin, remains unclear.

A redistribution of exogenously expressed wild type nucleolin from nucleoli to the nucleoplasm is observed when CHO cells are treated with the methyltransferase inhibitors 5-methyl 5' deoxythioadenosine (MTA) and 3-deaza-adenosine (DAA). This redistribution of nucleolin is reversible and independent of protein synthesis.



The arginines in the RGG motifs of the GAR domain of hamster nucleolin were changed to lysine, and the fully substituted protein was not a substrate for the RGG specific protein arginine methyltransferase, Hmt1p. The lysine substituted protein bound nucleic acids and behaved *in vivo* in a manner indistinguishable from wild type nucleolin. These results indicate that methylation is not necessary for *in vitro* nucleic acid binding or for *in vivo* localization of nucleolin, and that the redistribution of nucleolin observed following MTA treatment is likely due to inhibiting the methylation of another nucleolar substrate.

## **Chapter 1**

### **Literature Review**

Ribosomes are the biosynthetic engines that, convert the static sum of evolution in the form of DNA and its RNA intermediary into dynamic proteins that catalyze the unusual chemical reaction known as life. Eukaryotic ribosomes are perhaps the most complex macromolecular assemblies. They consist of four precisely folded and modified RNAs and over eighty different ribosomal proteins. The ribosome consists of two subunits that come together to form the functional complex. Protein synthesis requirements of the cell are dependent on ribosomes; it is therefore logical that ribosome biogenesis is regulated with regards to the metabolic state of the cell as well as its stage in the cell cycle. To successfully assemble this complex, transcription of the ribosomal RNAs and production of ribosomal proteins, as well as proteins involved in processing and assembly, must all be finely coordinated and brought to bear at the correct time and place. An actively growing HeLa cell, for example, synthesizes around 7500 ribosomal subunits per minute, using up to 300,000 ribosomal proteins in their assembly. Before assembly of the rRNA with these proteins, each pre-rRNA interacts with almost 150 different species of small nucleolar RNAs that guide various post-transcriptional modifications and cleavages. The site of all this activity is the nucleolus. In addition to its long standing role in ribosome biogenesis, the nucleolus has new potential functions, including post-translational RNA modification, ribonucleoparticle assembly, gene silencing, aging and cell cycle control (reviewed by Hadjiolov, 1985, Schwarzscher and Wachtler, 1993; Mèlèse and Xue, 1995; Shaw and Jordan 1995; Jordan, 1987; Pederson, 1998a; Pederson, 1998b, Garcia and Pillus, 1999; Lewis and Tollervey, 2000; Pederson,

2000, Schwarzscher and Mosgoelner, 2000; Olson et al., 2000; Dundr and Misteli, 2001; Leary and Huang, 2001).

## **THE NUCLEOLUS**

Due to its prominence when visualized by light microscopy, the nucleolus was one of the first sub-nuclear structures to be described by Fontana in 1774 and by R. Wagner, G. Valentin, and M. Schleiden between 1835-1838 (Miller, 1981). Its prominence is due to a very high protein concentration, which is typically the highest concentration in the cell. The number and shape of nucleoli vary depending on species, cell type, location in the cell cycle, and metabolic state of the cell (Hadjilov, 1985). Nucleoli are not delimited by a membrane; their existence appears to be the result of associations of components needed for ribosome biogenesis (Mélèse and Xue, 1995). The metazoan nucleolus is morphologically separated into three distinct concentric components, the inner fibrillar center (FC), the middle dense fibrillar component (DFC), and the outer granular component (GC). This concentric morphology of the nucleolus reflects the vectorial process of ribosome biogenesis.

### **Structure**

The morphology of the nucleolus varies depending on the organism, the cell type, and the location in the cell cycle. These differences are mainly associated with the metabolic state of the cell and its need for ribosomes. Quiescent cells usually have one compact nucleolus, typified by well defined concentric sub-domains. Active cells often have multiple nucleoli, that become larger and reticulated with increased metabolism (Schwarzscher and Mosgoelner, 2000).

The nucleolus is the location of tandemly repeated ribosomal genes (rDNA genes) collectively known as the nucleolar organizer region (NOR). Heitz (1931) and McClintock (1934) established the NOR as a genetically determined element on mitotic chromosomes that gave rise to interphase nucleoli. NORs were first identified as secondary constrictions present in the mitotic chromosomes that appeared to correspond to reforming nucleoli in teleophase in *Zea mays* (McClintock, 1934). The tandemly repeated arrays of ribosomal genes in the form of condensed mitotic chromatin are located on several “nucleolar chromosomes,” usually near one of the two telomeres.

The metazoan ribosomal DNA repeating transcription unit from 5' to 3' consists of an external transcribed spacer (ETS), 18S rDNA, internal transcribed spacer 1 (ITS 1), 5.8S rDNA, internal transcribed spacer 2 (ITS 2), 28S rDNA, all followed by an intergenic spacer region (IGS). The 5S rDNA gene lies within this same repeating unit in budding yeast, but 5' to the main rDNA gene. However, it is transcribed in the opposite orientation by RNA polymerase III rather than by RNA polymerase I. The 5S rDNA genes in metazoans are usually located on “non-nucleolar” chromosomes, and they are transcribed by RNA polymerase III. Transcription of the 5S rRNA is usually positioned in close proximity to the nucleolus, presumably to allow quick transport of the RNA to the nucleolus (Hadjiolov, 1985; Schwarzacher and Wachtler, 1993).

In interphase cells the fibrillar center appears as a light region under phase-contrast microscopy. Under electron microscopy, the FC appears as a meshwork of fine fibrils, 4-5 nm thick. The shape of the fibrils is roughly globular. FCs vary in size from 50 nm to 1  $\mu$ m. In quiescent cells with a compact nucleolus, there is one FC. As cells become active, however, multiple FCs become visible, but they eventually disappear

altogether, possibly as the result of chromatin decondensation and a release of transcription factors from the FC to accommodate the increased transcriptional requirements. Although some suggest that active transcription does not occur in the FC, transcription machinery including RNA polymerase I, upstream binding factor (UBF), selectivity factor 1 (SL1), topoisomerase I, DNA helicases and other factors related to transcription are localized to the FCs (Scheer and Rose, 1984; Rose et al., 1988, Jordan et al., 1996; Voit et al., 1995; Zirwes et al., 2000).

The DFC associates with or surrounds the FC. Early work indicated that the FC is the site of active ribosomal RNA transcription, but recent high resolution *in situ* hybridization with a 5'-ETS probe and Br-UTP incorporation studies reveal that active rDNA genes reside on the periphery of the fibrillar centers and are excluded from their interiors. The results localized rDNA transcription to the DFC, or at least to FC-DFC boarder (Thiry et al., 1992; Hozák et al., 1994; Puvion-Dutilleul et al., 1997; Mosgoeller et al., 1998; Cmarko et al., 1999; reviewed by Scheer and Hock, 1999; Raska et al., 1992). However, the debate over the site of ribosomal RNA transcription is still questioned by some (Mais and Scheer, 2000).

Processing cleavages leading to the formation of the mature 18S, 5.8S, and 28S rRNAs begin in the DFC. Post-transcriptional modifications such as pseudouridine synthesis and 2'-O-methylation of rRNA also occur in the DFCs (Nicoloso et al., 1996; Shaw and Jordan, 1995). Like the FC, the DFC consists of very fine fibrils of 3-5 nm in thickness, but the fibrils are very densely packed together giving them high electron microscopic contrast. In very active nucleoli, the fibrils appear to form sheets termed nucleolonema. The nucleolonema has been observed to possibly form a path for pre-

ribosomal particles within the nucleolus (Ghosh and Paweletz, 1996). The size of the DFC is usually directly related to nucleolar activity (Schwarzacher and Wachtler, 1993).

The GC is located at the periphery of the nucleolus and intermingles with the DFC. The GC is the site of maturing pre-ribosomal particles, consisting of small granules that measure 10-20 nm in diameter. Processing cleavages and post-translational modifications, along with assembly of ribosomal proteins onto rRNA that started in the DFC, are completed in the GC (Schwarzacher and Wachtler, 1993). In summary, the rRNA in the form of pre-ribosomal particles moves through the DFC to the GC by radial flow from the site of its transcription (Lazdins et al., 1997; Thiry et al., 2000).

When transcription of rRNA is inhibited by actinomycin D or by the onset of mitosis, metazoan nucleoli disassemble, indicating the need for active transcription to maintain nucleolar morphology (Phillips and Phillips, 1971; Recher et al., 1971; Scheer et al., 1975; Noel et al., 1971; Ploton et al., 1987; Weisenberger and Scheer, 1995; Dundr et al., 2000). Ectopic expression of rDNA in *Drosophila* resulted in the formation of nucleoplasmic micronucleoli (Karpen et al., 1988). The same was true for transcription of rRNA from a polymerase II promoter on a replicating plasmid in yeast (Oakes et al., 1998). These micronucleoli are able to produce functional ribosomes in the absence of morphologically normal nucleoli, supporting the idea that nucleoli are the result of associations of proteins and RNAs needed for ribosome biogenesis.

The disassembly of metazoan nucleoli at the onset of mitosis is accompanied by hyperphosphorylation of many of the non-ribosomal nucleolar proteins that are involved in ribosome biogenesis. Once phosphorylated by mitosis specific kinases (e.g. cyclin-dependent kinase I (CDK1)), the nucleolar proteins redistribute to the cytoplasm. RNA

polymerase I transcription is also halted in mitosis. The polymerase I transcription machinery, however, remains associated with the NORs during mitosis (Scheer and Rose, 1984; Roussel et al., 1996). Inhibition of polymerase I is mediated by CDK1; blocking CDK1 activity induces resumption of rDNA transcription in mitotic cells. Primary transcripts synthesized in mitosis remain unprocessed, thus indicating an independent mechanism for resumption of rRNA processing (Sirri et al., 2000). In contrast, partially processed pre-rRNA is found associated with a number of processing components, localized to the perichromosomal layer during mitosis, or in numerous large aggregates known as nucleolus derived foci (NDF) that are distributed throughout the mitotic cytoplasm (Ochs et al., 1985; Azum-Gélade et al., 1994; Pai et al., 1995; Dundr and Olson, 1998; Fomproix et al., 1998; Dundr et al., 2000).

In late anaphase, RNA polymerase I activity resumes, and pre-nucleolar bodies (PNBs) form as the processing components are released from the perichromosomal layer of decondensing chromosomes. These PNBs are nucleated by partially processed pre-rRNA. PNBs do not coalesce to reform nucleoli as previously thought; live cell imaging indicates that PNBs act as stores of nucleolar components. In teleophase the nucleolar components leave the PNBs and diffuse to nascent pre-rRNA transcripts at the re-activated nucleolar organizers to thus reform nucleoli. Interestingly, the order in which nucleolar components enter nucleoli corresponds to their position in the pre-rRNA processing pathway, reinforcing the idea presented by Mélése and Xue (1995) that the nucleolus results from of the “act of building a ribosome” (Dundr et al., 2000; Dundr and Misteli, 2001).

Although images of interphase nucleoli make them appear very stable, they are in fact very dynamic. Proteins needed for pre-rRNA processing or ribosome assembly only transiently associate with nucleoli, and they exchange rapidly with the surrounding nucleoplasm. Photobleaching experiments show that pre-RNA processing factors very rapidly exchange with the surrounding nucleoplasm. Photobleaching has shown the mean residence time is less than forty seconds for the non-ribosomal nucleolar protein fibrillarin (see below), and presumably the box C/D class of snoRNA's (see below) that associate with fibrillarin. The steady state concentration of fibrillarin, however, is clearly nucleolar in interphase cells. This is reflected by the fact that fibrillarin spends only four seconds in the nucleoplasm as compared to the roughly forty seconds in the nucleolus during a given cycle (Phair and Mistili, 2000; Snaar et al., 2000). Similar results have also been shown for other factors involved in ribosome biogenesis, including nucleolin, B23, and RNase MRP. In contrast, ribosomal proteins L9 and S5, are much slower in their nuclear mobilities (Chen and Huang, 2001). A possible function for the cycling of nucleolar proteins could be to expose nucleolar proteins to the nuclear factors that regulate their activity (e.g. CDK1, casein kinase II (CKII) that are non-nucleolar in their localization.

### **Function**

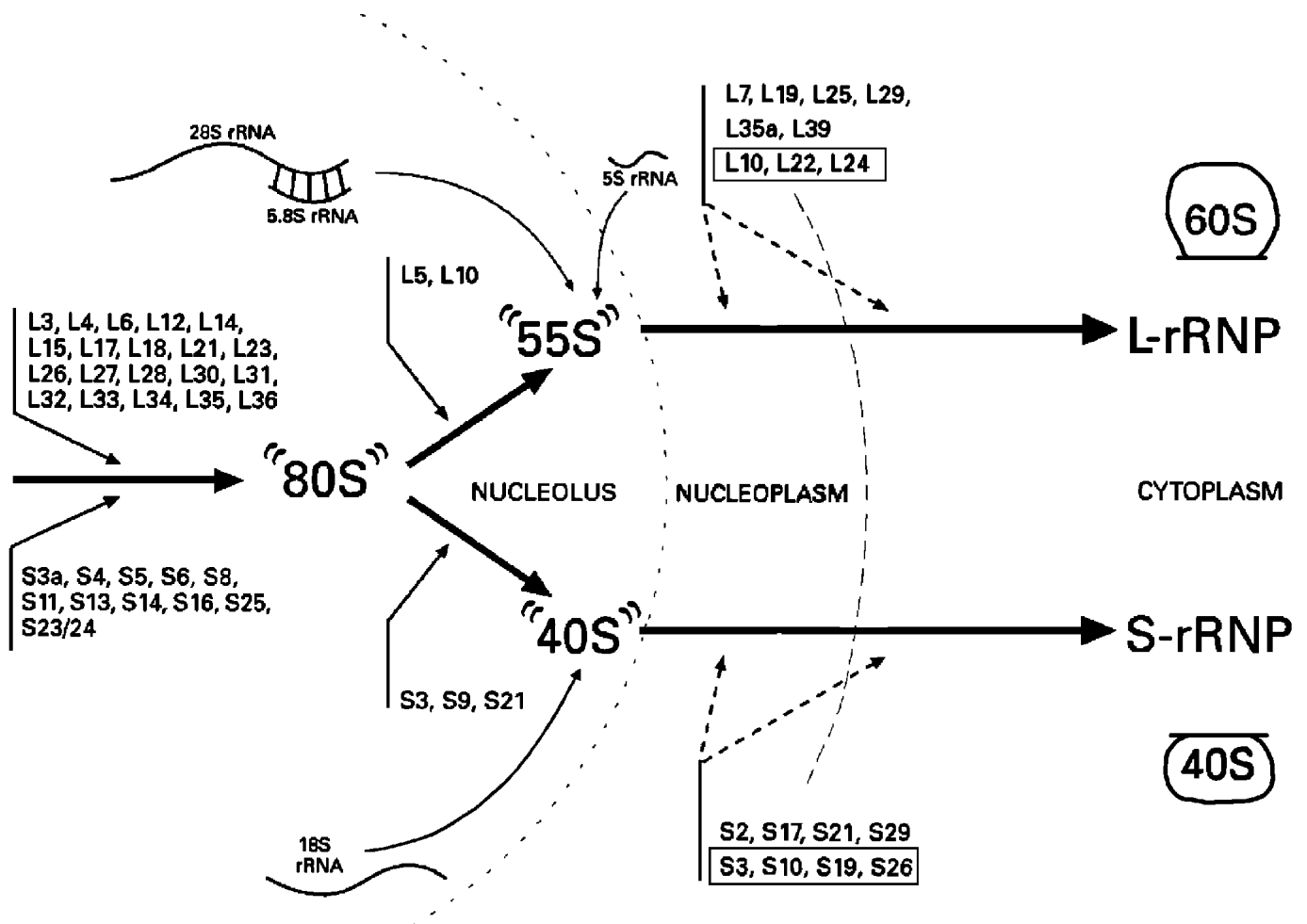
The major activity in the nucleolus is ribosome biogenesis. This includes the transcription of ribosomal RNA, the subsequent processing of the 47S (mammalian form) primary transcript into the mature 18S, 5.8S and 28S ribosomal RNA's, and the assembly with ribosomal proteins to form the pre-ribosomal particles that are then exported to the cytoplasm to form mature ribosomes. Biogenesis of the ribosome is perhaps one of the



most complex pathways of ribonucleoparticle synthesis in the cell. In eukaryotes it involves a complex rRNA maturation pathway and the ordered assembly of about 80 ribosomal proteins (see Fig. 1.1) (Hadjilov, 1985).

Ribosome biogenesis begins with transcription of the ribosomal RNA by RNA polymerase I. Transcription in eukaryotes is a complex process requiring concerted interactions between multiple protein factors, DNA elements, and RNA polymerase I. The promoter sequences for the ribosomal genes show little homology to even relatively closely related species, presumable due to rapid rate of evolutionary changes brought about by gene conversion, unequal crossing over, and excision-integration events that are enhanced by the repetitive nature of the ribosomal genes. These divergent promoter sequences are tolerated by a dedicated transcription machinery. Although the specific sequences are not well conserved, the overall structural organization of rDNA promoters from eukaryotes is comparable. The rDNA promoter consists of a start site proximal core promoter element (CPE) and distal elements, including the upstream control element (UCE), enhancers, terminators, and spacer promoters. The CPE is sufficient for faithful transcription initiation, while upstream elements stimulate promoter activity without affecting transcriptional specificity (Haltiner et al., 1986; Windle and Sollner-Webb, 1986; Mougey et al., 1996; reviewed by Grummt, 1999).

The CPE (~ -10 to -40) upstream of the transcription initiation start site is the binding location for the pre-initiation complex. First, promoter SL1 and UBF, bind to the CPE to recruit RNA polymerase I, as well as two polymerase I associated factors, TIF-1A and TIF-IC. This forms a productive initiation complex. Recent work by Ingrid Grummt's laboratory has helped elucidate the mechanism responsible for repression of



**Figure 1.1** Maturation of the eukaryotic ribosome. Structural large (L) and small (S) proteins are added to the 45S rRNA to form the 80S preribosome. The 55S preribosome is precursor to the mature 60S large ribosomal subparticle, and the 40S preribosome is precursor to the mature 40S small ribosomal subunit. Both the 60S and 40S particles are formed from the 80S preribosome through the addition of specific L and S proteins, respectively. Adapted from Hadjiolov (1985).

RNA polymerase I synthesis during mitosis. Inhibition begins in prophase and ends in late anaphase. Grummt's group showed that cdc2/cyclin B kinase (CDK1) phosphorylates SL1, impairing its interaction with UBF, and thus preventing formation of the pre-initiation complex (Heix et al., 1998; Kuhn et al., 1998). Upstream enhancers seem to stimulate transcription via stabilization of the pre-initiation complex (Pape et al., 1990; Xie and Rothblum, 1992). The RNA polymerase I initiation complex is stable for several rounds of transcription (Sollner-Webb and Mougey, 1991). During mitosis, initiation complexes are believed to be primed on rDNA promoters awaiting decondensation of chromatin, which might loop out into the DFC to begin active transcription as mitosis draws to an end (Grummt, 1999). Additional components such as topoisomerase I, single stranded binding protein (SSB), and helicases like NOH61 are present on the nucleolar organizer and needed for transcription (Zirwes et al., 2000; Weisenberger et al., 1993).

The rate of rDNA transcription is regulated in accordance with cell growth, and the need for new ribosomes. When cells approach a stationary phase or are serum-starved, rDNA transcription is down-regulated. Conversely, rDNA transcription is up-regulated upon reversal of such conditions (Buttgereit et al., 1985; Paule et al., 1984; Klein and Grummt, 1999).

Further control of ribosome biogenesis is due in part to the interconnections between various nucleolar factors. For example, several of the introns of pre-mRNAs that encode some ribosomal proteins as well as ribosome assembly factors like nucleolin (see below), contain snoRNAs needed for pre-rRNA processing (Caffarelli et al., 1994; Liang-Hu et al., 1995). Subsequently, nucleolin is regulated by transcription factors such

as *myc* and *myb*, which are associated with cellular metabolic regulation. *Myc* and *myb* are targets of cell cycle regulatory proteins such as CDK1. Thus the regulation of *myc* and *myb* ties nucleolin and ribosome biogenesis into growth regulation (Belenguer et al., 1989; Greasley et al., 2000; Ying et al., 2000).

Transcription of mammalian ribosomal DNA genes yields a 47S primary transcript, (~13,000 nt in humans), which is fully transcribed before any processing cleavage occurs (Steitz and Tycowski, 1995; Grummt, 1999). Processing of the 47S primary transcript yields three mature ribosomal RNAs (18S, 5.8S, and 28S). Coordinate expression of the three mature rRNAs is accomplished by their existing in a single transcript allowing 1:1:1 ratios of the mature rRNAs to be produced. The 5S rRNA is transcribed by RNA polymerase III outside the nucleolus. The expression of 5S rRNA is coordinated with the expression of the other ribosomal rRNAs; however, it is usually present in excess within the cell as compared to the other rRNAs. This is probably to prevent production of the 5S rRNA being the limiting factor in ribosome biogenesis (Hadjiolov, 1985).

The pre-rRNA maturation pathway varies among eukaryotic species as well as with growth and development in the same species. The processing pathway in mouse cells is “typical” for other vertebrate processing pathways, and it has been well studied using in vitro processing lysates. In the following description, “cleavage” refers to a particular site, but it has not been definitely established in many cases whether the “cleavage” is due directly to an endonucleolytic cleavage or whether the cleavage actually results from the trimming action of an exonuclease that initiated its attack from some other endonucleolytic site (reviewed by Eichler and Craig, 1994).

The initial pre-rRNA transcript is 47S. It is rapidly cleaved in the 5' external transcribed spacer (ETS) (+650 upstream of the 18S rRNA in mouse, +414 in humans). This initial event is known as the primary processing cleavage (Kass and Sollner-Webb, 1987; Kass and Sollner-Webb, 1990; Kass et al., 1990; Craig et al., 1991). Following this initial ETS cleavage (or coordinate with it), the 3' end of the 28S rRNA is cleaved to yield the 45S intermediate. The next cleavage occurs at the ETS-18S boundary yielding the 24S ETS RNA which is rapidly degraded, and the 41S downstream intermediate. Cleavage within the 41S pre-rRNA at the 18S-ITS1 boundary yields the mature 18S rRNA and a 36S intermediate. The 36S intermediate in turn is cleaved at its ITS1-5.8S boundary releasing ITS1 to be degraded, and a 32S intermediate. The 32S intermediate contains the 5.8S-ITS2-28S rRNA; it is cleaved at the ITS2-28S boundary to yield mature 28S rRNA and a 12S intermediate. This last processing event is well conserved, and it appears to be the only pathway for processing the 32S intermediate. Finally, the remaining 12S intermediate is cleaved at the ITS2-5.8S boundary to yield the mature 5.8S rRNA. The 5.8S and 28S rRNAs are hybridized over a portion of their single stranded regions before processing cleavages are completed, and this relationship is maintained in the mature 60S (large) ribosomal particle. The 5S rRNA, synthesized outside the nucleolus, requires very little processing as the primary transcript contains only a few extra nucleotides at its 3'-end. The 5S rRNA either diffuses or is carried to the nucleolus by the La autoantigen (Wolin and Matera, 1999).

Some RNA processing events, such as those involved in mRNA splicing, must be extremely precise to maintain the proper codon reading frame. It is not clear, however, if rRNA processing requires the same level of precision, since there is no reading frame to

maintain. Limited heterogeneity of a few nucleotides has been reported for the ends of 5.8S and 28S rRNAs in functioning ribosomes. Cleavages and trimming reactions may not be exact, but rather only approximate (Bowman et al., 1983; Eichler and Craig, 1994).

Ribosomal and non-ribosomal proteins associate with the primary transcript before transcription is completed. Thirty nanometer structures known as terminal balls are clearly visible on growing primary transcripts in Miller spreads of actively transcribed rDNA (Mougey et al., 1993). These terminal balls form on the ETS and are intimately involved in the initial cleavage that occurs in the ETS. Processing is concurrent with assembly of the pre-ribosomal particle. Ribosomal RNA intermediates do not exist as free RNA, but instead as ribonucleoprotein complexes. The 5.8S, 28S and 5S rRNAs associate with approximately 49 large-subunit ribosomal proteins that together form the large 60S ribosomal particle. The 18S rRNA and approximately 33 small-subunit ribosomal proteins form the small 40S ribosomal particle (see Fig. 1.1). Mature ribosomal subunits are exported to the cytoplasm by specific nucleoplasmic receptors and some members of the karyopherin family in an energy dependent process (Bataille et al., 1990; Aitchison and Rout, 2000).

In yeast, the processing scheme is basically similar to that just described for mammals, except that the primary transcript is a 35S pre-rRNA, and the 28S mature rRNA normally found in metazoans is only 25S in yeast. Because the rate of pre-rRNA processing is more rapid in yeast than in mammals, cleavages at the 5' ETS-18S and ITS1-5.8S boundaries appear to occur simultaneously. Therefore, alternative pathways are not detectable under normal growth conditions in wild type yeast cells. There are also

fewer ribose methylations and pseudouridines present in mature yeast rRNA (Eichler and Craig, 1994; Filipowicz et al., 1999). Other processing events also occur in certain organisms. For example, in *Tetrahymena*, there is a group I intron present within the 27S rRNA sequence that is spliced out during processing. Some organisms including *Drosophila*, *Sciara*, *Euglena*, some annelids, as well as mollusks remove small segments known as hidden breaks (roughly 19 nucleotides long) from their 28S rRNA during processing (Eichler and Craig, 1994).

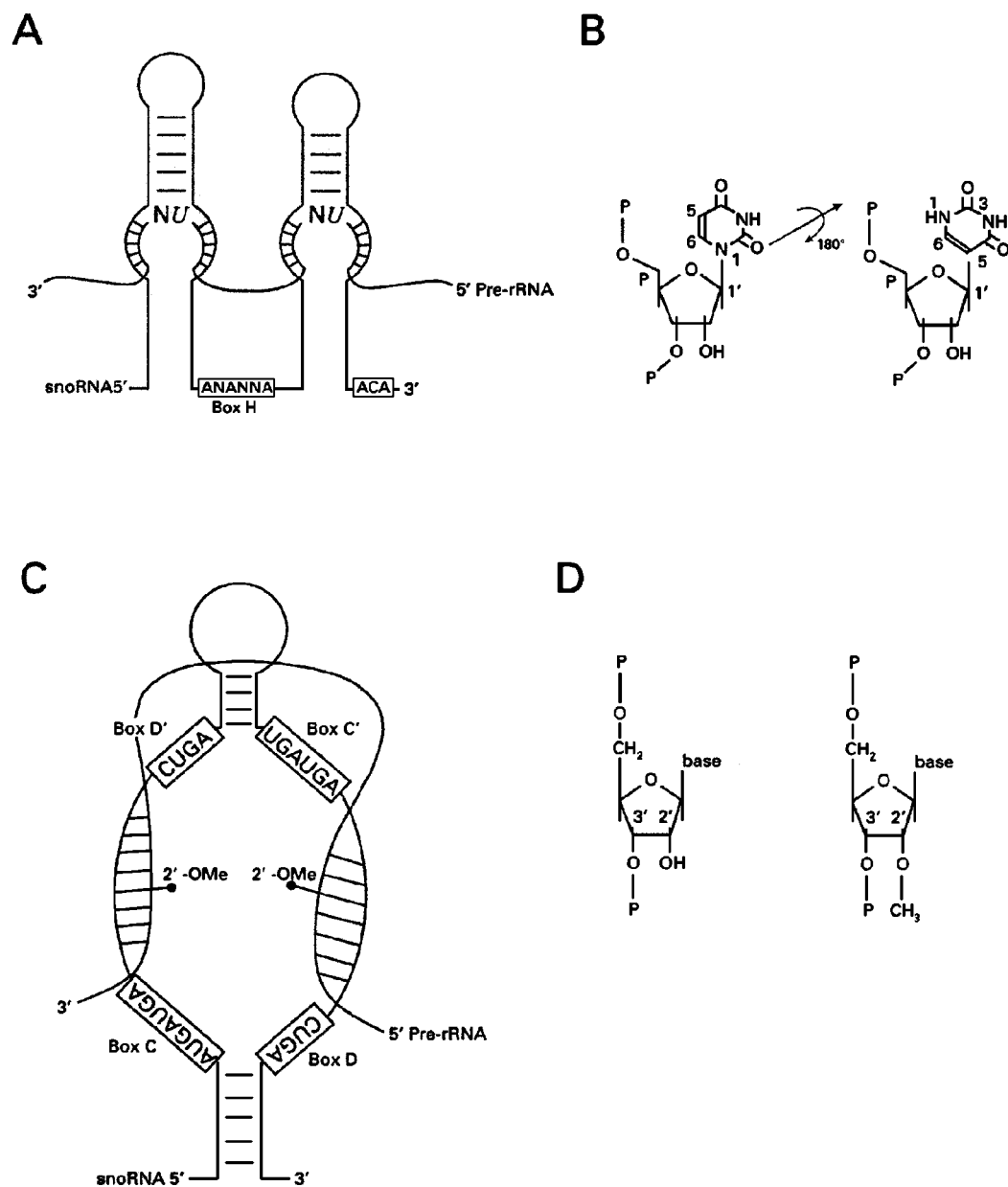
The initial cleavage event within the 5' ETS terminal balls involves fibrillarin, nucleolin and the small nucleolar RNA, U3 (Kass et. al, 1990; Turley et al., 1993; Ginisty et al., 1998). Other factors implicated in other ribosomal rRNA processing cleavages include snoRNPs U8, U13, U14, U17, U20, E2, E3, snR10\*, snR13, snR30\*, RNase MRP, RNase PR1\* and the proteins fibrillarin, Nop56, Gar1p\*, NOP2\*, and NOP3\* (Bennett and Clayton, 1990; Karwan et al., 1991; Peculis and Steitz, 1993; Steitz and Tycowski, 1995; Cavaille et al., 1996; Meier, 1996; Ochs et al., 1996). The \* designates a yeast protein with no identified metazoan homolog. The roles played and the mechanisms of action of these factors remain unclear. Recently, yeast factors responsible for the endonucleolytic cleavages and exonucleolytic trimming reactions responsible for producing the mature rRNAs were found to exist as a large complex, called the exosome. The exosome in yeast contains at least 10 proteins (Rrp4p, Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p, Mtr3p, Rrp44p/Dis3p, Rrp4p, Rrp40p), and it is required for maturation of the 18S, 5.8S and 25S rRNAs (Allmang et al., 1999; Zanchin and Goldfarb, 1999). The human homolog of the yeast exosome, PM-Sci 100, has been localized to nucleoli of HeLa cells (Fomproix and Hernandex-Verdun, 1999; Mitchell and Tollervey, 2000;

Brouwer et al., 2001). The mechanism directing cleavages is thought to involve primary sequences as well as the secondary structures of rRNA (Savino et al., 2001).

Processing also includes a series of specific base modifications, namely ribose 2'-O-methylation and pseudouridine conversion (reviewed by Bachellerie et al., 1995; Maxwell and Fournier, 1995; Beven et al., 1996; Tollervey, 1996; Edward et al., 1997; Weinstein and Steitz, 1999). The ribose 2'-O-methylation reactions are directed by the box C/D snoRNPs. These RNPs contain small nucleolar RNAs that contain the conserved C box (RUGAUGA; where R is any purine) and D box (CUGA) elements near their 5' and 3' termini, respectively. Internal C/D box sequences have also been identified (Box C' and D') (Kiss- László and Kiss 1996; Kiss- László and Kiss, 1998). The box C/D snoRNAs contain small guide sequences that are complementary to the ribosomal RNAs. These guide sequences form short duplexes within the rRNA (10-21 nt in length). Duplex formation allows the snRNAs to act as guides for the methylation machinery. The fifth residue upstream of the D box or D' box is methylated at the 2' position of the ribose ring (see Fig.1.2). The proteins catalyzing the methylation reaction are not known, but both fibrillarin and p120 associate with box C/D snoRNAs, and both may have a S-adenosyl methionine (SAM) binding site (Freeman et al., 1989; Koonin, 1994; Wang et al., 2000).

The other class of snoRNAs, the box H/ACA RNAs, direct the pseudouridylation reaction. The Box H/ACA snoRNAs have a conserved hairpin-hinge-hairpin-tail secondary structure that is required to direct pseudouridine conversion. Box H (ANANNA, where N is any nucleotide) is found within the hinge region, whereas box H/ACA lies 3 nucleotides from the 3' end of the guide RNA. Box H/ACA snoRNAs base





**Figure 1.2** The box H/ACA and box C/D snoRNAs. **A** The predicted structure of the hybrids between a box H/ACA snoRNA and the pre-rRNA at the sites of pseudouridine (Ψ) formation. The sequences flanking the site of modification are base-paired to the snoRNA in a complex pseudoknot structure, while the base that is to be modified by rotation about the N<sub>3</sub>-C<sub>6</sub> axis is free to interact with the Ψ synthase. The base pairing involves two stretches of 3-10 nucleotides on either side of the base to be modified. **B** Ψ formation by base rotation. **C** The predicted structure of the hybrids between a box C/D snoRNA and the pre-rRNA. The box C/D and/or C'/D'-pre-rRNA interaction generates a conserved structure with the box D or D' element placed five base pairs downstream from the site of 2'-O-methylation. **D** 2'-O-methylation of the ribose sugar moiety. Used with permission Waggener 2001©

pair with their target pre-rRNA site to form two duplexes, forming a pseudouridylation pocket 14-17 nucleotides upstream of box H or ACA. The unpaired uridine in the pocket is converted to pseudouridine (see Fig. 1.2) (Ganot et al., 1997; Ni et al., 1997; Bortolin et al., 1999). The proteins dyskerin and Nopp140 are known to associate with box H/ACA snoRNAs (Mitchell et al., 1999).

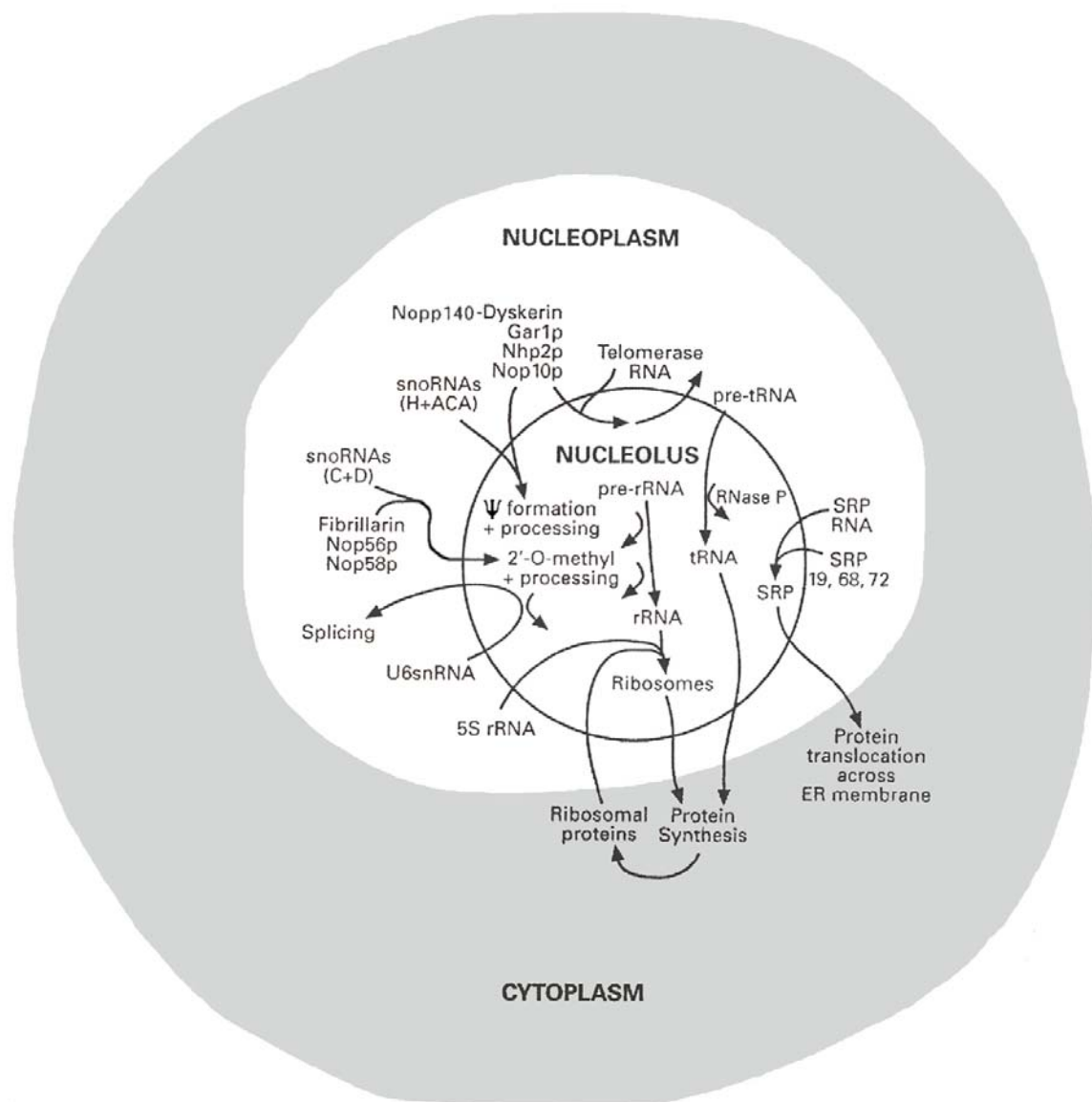
Although the functional significance of the post-transcriptional modifications are not fully understood, methylated and pseudouridylated residues are confined to the most highly conserved portions of the mature rRNA sequences found within the ribosome. These modified sites also correspond to the residues associated with the active site of the ribosome (Lewis and Tollervey, 2000). An additional potential hydrogen bond at the N-1 position is created by pseudouridine conversion, which may contribute to rRNA folding and contribute to overall stability in a manner similar to pseudouridine in tRNAs (Durant and Davis, 1999). Ribose methylation may stabilize rRNA by increasing hydrophobic interaction surfaces (Smith and Steitz, 1997). The physical interaction with snoRNAs themselves may alter the architecture of pre-rRNA and thus achieve proper folding of the mature rRNA molecule (Lewis and Tollervey, 2000).

Processing and assembly of the pre-ribosomal particles involves ribosomal as well as non-ribosomal proteins. From the moment that the pre-rRNA transcript comes off polymerase I, it associates with proteins (see above). Non-ribosomal nucleolar proteins that localize in a ring around the FC-DFC boarder are thought to be some of the early associating proteins (Shah et al., 1996; Waggner and DiMario 2001). They include Nopp140 and fibrillarin, which are implicated in pseudouridine synthesis and 2'-O-methylation respectively, as well as a host of functions. Conversely, B23 and nucleolin

are localized throughout the DFC and partially into the GC. B23 and nucleolin are believed to associate early in pre-rRNA processing, but then remain associated with the RNA until late assembly, perhaps playing a role in the vectorial movement of ribosomal particles through the nucleolus and the export of the completed ribosomal subunits to the cytoplasm, as well as a host of other possible functions (see below).

### **Novel Nucleolar Functions**

In addition to its main role as the site of ribosome biogenesis, recent work suggests that the nucleolus is a major site of other RNA modifications, and that it plays roles in gene silencing, aging and cell cycle control (reviewed by Pederson, 1998b; Garcia and Pillus, 1999). Certain small RNAs, destined to be components of ribonucleotide complexes, transiently associate with the nucleolus to undergo modification (see Fig 1.3). For example, the signal recognition particle (SRP) RNA has been shown to transit through the nucleolus. Experiments showed that fluorescently label SRP RNA, microinjected of into a mammalian cell nucleus, rapidly accumulated in the nucleolus before exiting to the cytoplasm. Additionally, three of the six proteins found in the mature SRP particle have been localized to the nucleolus (Jacobson and Penderson, 1998; Politz et al., 2000; Grosshans et al., 2001). Telomerase RNA, a functional component of another ribonucleoprotein (telomerase), is also directed to the nucleolus via sequences on its 3' end. The splicing small nuclear RNA, U6, localizes to the nucleolus where it undergoes 2'-O-ribose methylation (Tycowski et al., 1998; Lange and Gerbi, 2000; Ganot et al., 1999). The RNA component of human and yeast RNase P has also been localized to the nucleolus. RNase P is involved in maturation of tRNAs. Further, isopentenyl-6-adenosine synthetase generates isopentenyl-6-adenosine, which is found



**Figure 1.3** The RNA pathways within the nucleolus. Adapted from Lewis and Tollervey (2000).

only in tRNAs. Interestingly, isopentenyl-6-adenosine synthetase is localized to the nucleolus, indicating that the nucleolus is the site of tRNA maturation (Pederson, 1998b). Thus the nucleolus may serve as a general center for the modification and processing for multiple gene transcripts other than ribosomal RNAs.

### **NON-RIBOSOMAL NUCEOLAR PROTEINS**

The nucleolar protein p120 (Nol1, NOP2\*) was originally identified in human tumor cells by use of monoclonal antibodies (Witte et al., 1978). Its abundance closely associates with cell proliferation, and it serves as a useful histological marker for tumor cell proliferation (Uchiyama et al., 1997). Vertebrate p120 binds rRNA with a dissociation constant of 4 nM and is localized to the DFC which is consistent with a role in ribosome biogenesis (Gustafson et al., 1998). Deletion of the yeast homolog, NOP2, leads to impaired processing of the 35S pre-rRNA; mature 25S and 5.8S rRNAs form, but not the 18S rRNA. There is also a reduction in rRNA methylation (Hong et al., 1997).

Nopp140 (SRP40\*, p130) was first cloned from rat using an assay looking for proteins that interacted with the nuclear localization signal (NLS) of the SV40 large T antigen (Meier and Blobel, 1990). Nopp140 is a nucleolar phosphoprotein of 140 kDa, and it consists almost entirely of alternating acidic and basic domains. The C-terminus is highly conserved compared to the rest of the protein with a 59% identity between rat and yeast. Recently, a splice variant was identified in *Drosophila melanogaster* that contained a glycine and arginine rich carboxy domain instead of the evolutionary conserved carboxyl terminus (Waggner and DiMario, 2002). Vertebrate Nopp140 localizes to Cajal bodies and the DFC of the nucleolus, often in a ring around the FC. This staining pattern may reflect its interaction with RPA194 (the largest subunit of RNA

polymerase I) (Miau et al. 1997). Similar to several other nucleolar proteins, Nopp140 has both CKII and CDK1 recognition motifs, and it is highly phosphorylated during interphase (CKII) and mitosis (CDKI). Like other nucleolar proteins, Nopp140 shuttles between the nucleus and cytoplasm (Meier and Blobel, 1992). Shuttling is faster for Nopp140 than other nucleolar proteins, and Nopp140 appears to travel along tracks in the nucleus as determined by electron microscopy. This, together with the ability of Nopp140 to bind nuclear localization signals, suggests a possible role in the transport of nucleolar components (Meier and Blobel, 1992). Nopp140 is a component of both box C/D and box H/ACA snoRNPs, and it may have a role in transport of snoRNPs to the nucleolus (Yang et al., 2000).

Identified by its reaction with autoimmune antisera, fibrillarin (NOP1\*, B36) is a 34 kDa nucleolar protein rich in asymmetric  $N^G, N^G$  dimethylarginine (aDMA) (Lischwe et al., 1985). Fibrillarin is a conserved non-ribosomal nucleolar protein with clear homologues from yeast to humans (Aris and Blobel, 1991; Tollervey et al., 1993). Human fibrillarin consists of a well defined amino terminal glycine arginine rich (GAR) domain, two central RNA binding domains (RBDs) and a carboxy domain that may form alpha helices (Christensen and Fuxa, 1988; Aris and Blobel, 1991). In the nucleolus, fibrillarin localizes to the DFC, often in a ring around the FC. It is known to be involved in the primary pre-RNA processing reactions (cleavage and methylation) in vertebrates. Temperature sensitive mutations of NOP1 in yeast cause a decrease in methylation as well as impaired maturation of all rRNAs generated from the primary transcript at the non-permissive temperature. These processing defects may be indirectly related to its potential role in methylation. Fibrillarin is known to be associated with all box C/D

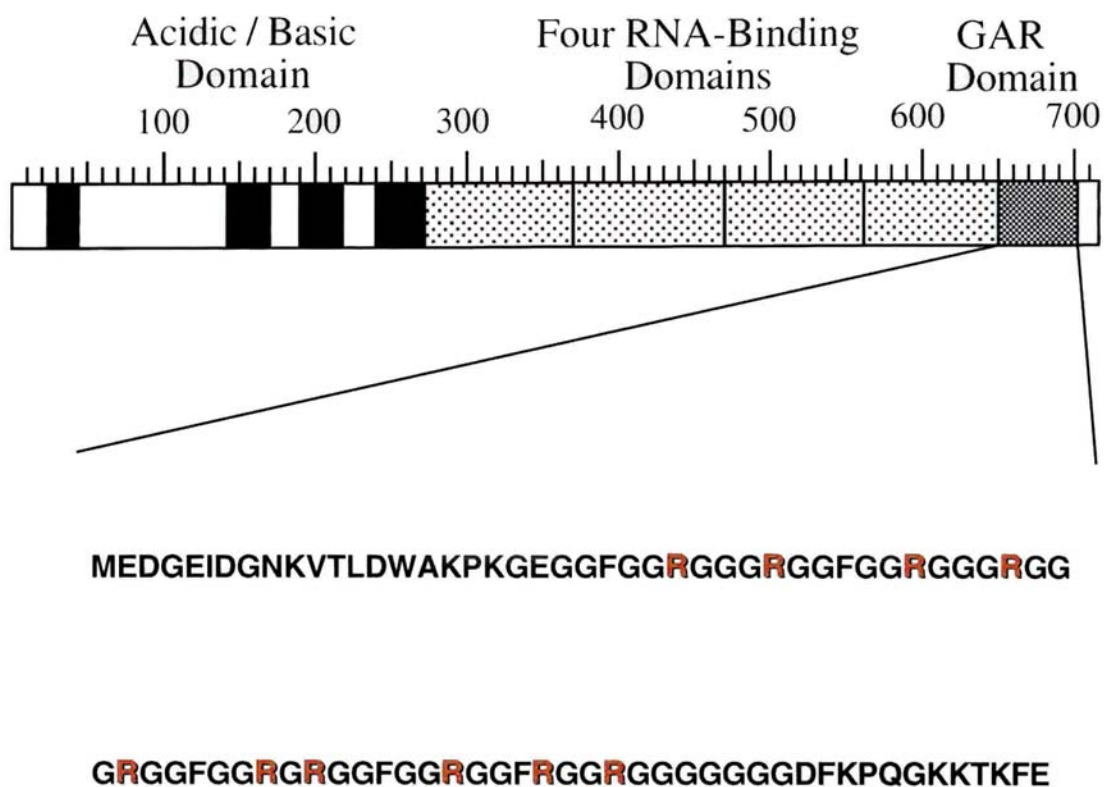
snoRNAs, and it shares structural homology with an archean bacterial methyltransferase (Tollervey et al., 1993; Wang et al., 2000).

B23 (numatrin, NO38, nucleophosmin) is a multifunctional nucleolar protein, often referred to as a putative ribosome assembly factor. It is present in both the DFC and GC (Ochs et al. 1983; Biggiogera et al. 1991). Its amino terminal end consists of repeating acidic and basic domains, similar to other nucleolar proteins (Nopp140 and nucleolin). It can be phosphorylated by CKII, CDK1, and the CDK2/cyclin E heterodimer. Although it lacks a consensus RBD, B23 can bind nucleic acids. In doing so, B23 displays helix destabilizing properties and ribonuclease activities (Dumbar et al., 1989; Herrera et al., 1995). The nucleic acid binding properties are associated with its carboxyl terminus. B23, however, is predominantly a hexamer *in vivo*, and the conditions that promote oligomerization *in vitro* abolish nucleic acid binding. B23's nucleic acid binding (apparent binding constant  $K_{\omega} = 50 \text{ nm}$ ) exhibits a very high cooperativity factor ( $\omega$ ) of 800, thus indicating that multimers may form on the DNA (Chan and Chan, 1995; Herrera et al. 1996; Wang et al., 1994; Dumbar 1989). There are two expressed variants of B23 in humans. The two forms are generated by alternative splicing of the mRNA; B23.1 is the major form, while B23.2 which lacks the last two exons, is localized primarily in the cytoplasm (Chang and Olson, 1989; Chang and Olson 1990). B23 has been identified as a *myc*-responsive gene due to *myc*-binding sequences in its first intron. B23 can relieve transcriptional repression caused by the YY1 repressor, which was shown to specifically bind the promoter region of the B23 gene. Responsiveness to the *myc* transcription factor, the ability to relieve YY1 repression, and its ability to stimulate DNA polymerase  $\alpha$ , together suggests a possible role in

transcriptional regulation (Takemura et al. 1994; Inouye and Seto, 1994; Chan et al. 1997; Umekawa et al., 2001; Zeller et al., 2001). B23 shuttles between the nucleolus and cytoplasm, and it has been implicated in the nucleolar localization of the viral Rev and Rex proteins via binding of their NLS signals. B23 binds only SV40 type NLS sequences, and thus binding is enhanced by CKII phosphorylation of B23. There is no apparent binding of B23 to bipartite NLS sequences (Fankhauser et al., 1991; Szebeni et al., 1995). In a yeast two-hybrid assay, B23 was shown to interact with nucleolin (see below). Taken together with its localization, nucleic acids binding properties, and ribonuclease activities, B23 is strongly implicated in ribosome biogenesis (Adachi et al., 1993; Fankhauser et al., 1991; Li et al., 1996). Finally, B23 has been shown to play an important role in regulation of centrosome duplication. During mitosis, B23 redistributes to the spindle poles (centrosomes) via hyperphosphorylation, and it is released only upon dephosphorylation. Phosphorylation of threonine 199 by CDK2/cyclin E in late G1 of the cell cycle causes dissociation of B23 from the spindle poles, which is critical for initiation of centrosome duplication (Okuda et al. 2000; Tokuyama et al., 2001).

Nucleolin (C23, NSR1\*, GAR2\*) is a major nucleolar phosphoprotein that constitutes as much as 10% of the nucleolar protein in actively growing cells. It has been implicated in rDNA transcription, rRNA processing, ribosome assembly and nucleocytoplasmic transport (Lapeyre et al., 1987; reviewed by Tuteja and Tuteja, 1998; Ginisty et al., 1999; Srivastava and Pollard, 1999). The amino terminus consists of alternating acidic / basic domains (see Fig 1.4). The basic domains contain CDK1 phosphorylation sites, and the acidic domains contain several CKII and protein kinase C $\zeta$





**Figure 1.4** A schematic diagram of Chinese hamster nucleolin as derived from the cDNA sequence of Lapeyre et al. (1987). The amino-terminal one-third consists of alternating acidic (*black boxes*) and basic (*open boxes*) domains. The carboxy-terminal two-thirds consists of four RNA-binding domains and the glycine and arginine rich (GAR) domain near the C-terminus. The GAR domain is expanded to show the peptide sequence.

phosphorylation sites (Belenguer et al., 1989; Peter et al., 1990; Li et al., 1996; Zhou et al., 1997)

The function of the amino terminus remains unknown. Nucleolin has been shown to interact directly with snoRNA U3 to promote the primary processing reaction (Yang et al., 1994; Ginisty et al., 1998; Ginisty et al., 2000). This interaction is mediated by the amino terminal third. On the other hand, the acidic domains of acidic amino terminus have been proposed to bind histone H1 to perhaps displace H1 from the linker DNA causing chromatin decondensation. The basic stretches within nucleolin contain an octapeptide repeat that bears strong similarity to histone H1, and those repeats could be responsible for nucleolin's ability to modulate DNA condensation in chromatin (Olson and Thompson, 1983; Erard et al., 1988; Erard et al., 1990; Kharrat et al., 1991).

Nucleolin has a bipartite nuclear localization signal (NLS) located between the amino terminal third and the four down-stream RNA binding domains (RBDs). This NLS is necessary and sufficient for nuclear accumulation, but not nucleolar accumulation, which appears to be the result of interaction with rRNA (Schmidt-Zachmann and Nigg, 1993; Créancier et al., 1993; Meßmer and Dreyer, 1993) (see below). Down-stream of the NLS are the four consensus sequence RBDs that are responsible for nucleolin's nucleic acid binding ability (see Fig.1.4). Deletion analysis, however, shows that RBDs 1 and 2 are responsible for nucleolin's high affinity sequence specific binding (Serin et al., 1997; Ginisty et al., 2001). SELEX experiments identified a RNA sequence that nucleolin recognizes with high affinity, ( $K_d = 5 \text{ nM}$ ). The RNA sequence consists of an 18 nt stem loop structure, with six nucleotides (UCCCGA) in the loop. Sequence comparisons and mutagenesis allowed characterization of a nucleolin

binding site with a 5 nt stem and a 7-10 nt loop containing the motif (U/GCCCGA). This constitutes the Nucleolin Recognition Element (NRE). Sequences similar to NRE have been located in the 5' ETS of mouse pre-rRNA near the primary cleavage site. This is consistent with nucleolin's involvement with this cleavage (Ghisolfi et al., 1992a; Ghisolfi et al., 1992b; Ghisolfi-Nieto et al., 1996; Allain et al., 2000).

Additionally, nucleolin can interact with G-G base paired rDNA that is able to form planar rings. These unusual structures known as G quartets are stabilized by Hoogsteen bonding between the G's. Nucleolin binds these structures with very high affinity ( $K_d = 1$  nM). G quartets have not been observed *in vivo*, but they can form readily and stably *in vitro*. No functional significance can be attributed to this nucleolin interaction, although it is the highest affinity interaction yet shown for nucleolin. Runs of guanines capable of forming G quartets when single stranded are prevalent in rDNA. The rDNA exists in single-stranded form during replication or transcription and this might promote formation of these G quartets. Nucleolin might modulate these G quartets if they exist. This concept is consistent with nucleolin's helicase activity (see below), highly specific binding, transcriptional regulation and nucleolar localization (Egyhazi et al., 1988; Tuteja et al., 1995; Hanakah et al., 1999).

Near nucleolin's carboxy terminus is a glycine arginine rich (GAR) domain defined by Arg-Gly-Gly (RGG) repeats interspersed with aromatic (particularly phenylalanine) residues (see Fig. 1.4). Removal of this domain abolishes nucleolin's non-specific ( $K_d \sim 100$  nm) nucleic acid binding, as well as its nucleolar localization, presumably through loss of *in vivo* nucleic acid binding. Schwab and Dreyer (1997) showed that RNase treatment induces the release of nucleolin from nucleoli in A6 cells,

thus supporting the idea that nucleolin localizes to nucleoli by binding pre-rRNA.

Separately, the GAR domain by itself shows low affinity RNA binding (see below) ( $K_d \sim 500$  nM). Strand annealing and limited ATP-dependent helix destabilizing (helicase) properties are attributed to the GAR domain, and they may be responsible for the GAR domain's ability to modulate nucleolin's interaction with RNA (Ghisolfi et al., 1992a; Heine et al., 1993; Hanakah et al., 1999; Hanakah et al., 2000), possibly by disrupting secondary structures of the RNA to thus allow access of the upstream RDBs.

Nucleolin, like other nucleolar proteins, is known to shuttle between the nucleus and cytoplasm. Nucleolin can also bind a subset of ribosomal proteins, thus implicating it in nucleocytoplasmic transport of these ribosomal proteins. The interaction is only with a subset of ribosomal proteins, and is mediated by either nucleolin's N-terminus or its GAR domain. Several of these ribosomal proteins are known to be internally located in mature ribosomal subunits, thus indicating an early association of these ribosomal proteins with pre-rRNA. Nucleolin has been shown to associate early with nascent pre-rRNA (Herrera and Olson, 1986). Knockouts of yeast nucleolin-like proteins, Gar2p from *Schizosaccharomyces pombe* and Nsr1p from *Schizosaccharomyces cerevisiae*, lead to accumulations of the 35S pre-rRNA, decreases in mature 18S rRNA, and an absence of free 40S ribosomal subunit (Gulli et al., 1995; Kondo and Inouye, 1992). Its early association with pre-rRNA, its location in the DFC (with some in the GC), and its specific binding of ribosomal proteins, all strongly implicate nucleolin in ribosome biogenesis. Current thinking portrays nucleolin as a RNA chaperone, modifying or maintaining specific rRNA secondary structures to allow correct processing and/or

assembly of ribosomal proteins with the pre-rRNA (Beyer et al., 1977; Escande et al., 1985; Borer et al., 1989; Bouvet et al., 1998).

Nucleolin has also been implicated in a number of non-traditional roles for a nucleolar protein. These include immunoglobulin gene switching, binding telomeric DNA, response to insulin, acting as a glucocorticoid receptor, a transcription factor for the ribosomal RNA genes, and even extracellular binding of an anti-HIV peptide at the cell surface (Csermely et al. 1993; Hanakahi et al., 1997; Nisole et al. 1999; Pollice et al., 2000; Schulz et al., 2001). The biological significance of these interactions is unknown, and they will have to await functional studies to discern their *in vivo* significance, if any.

## **PROTEIN ARGININE METHYLATION**

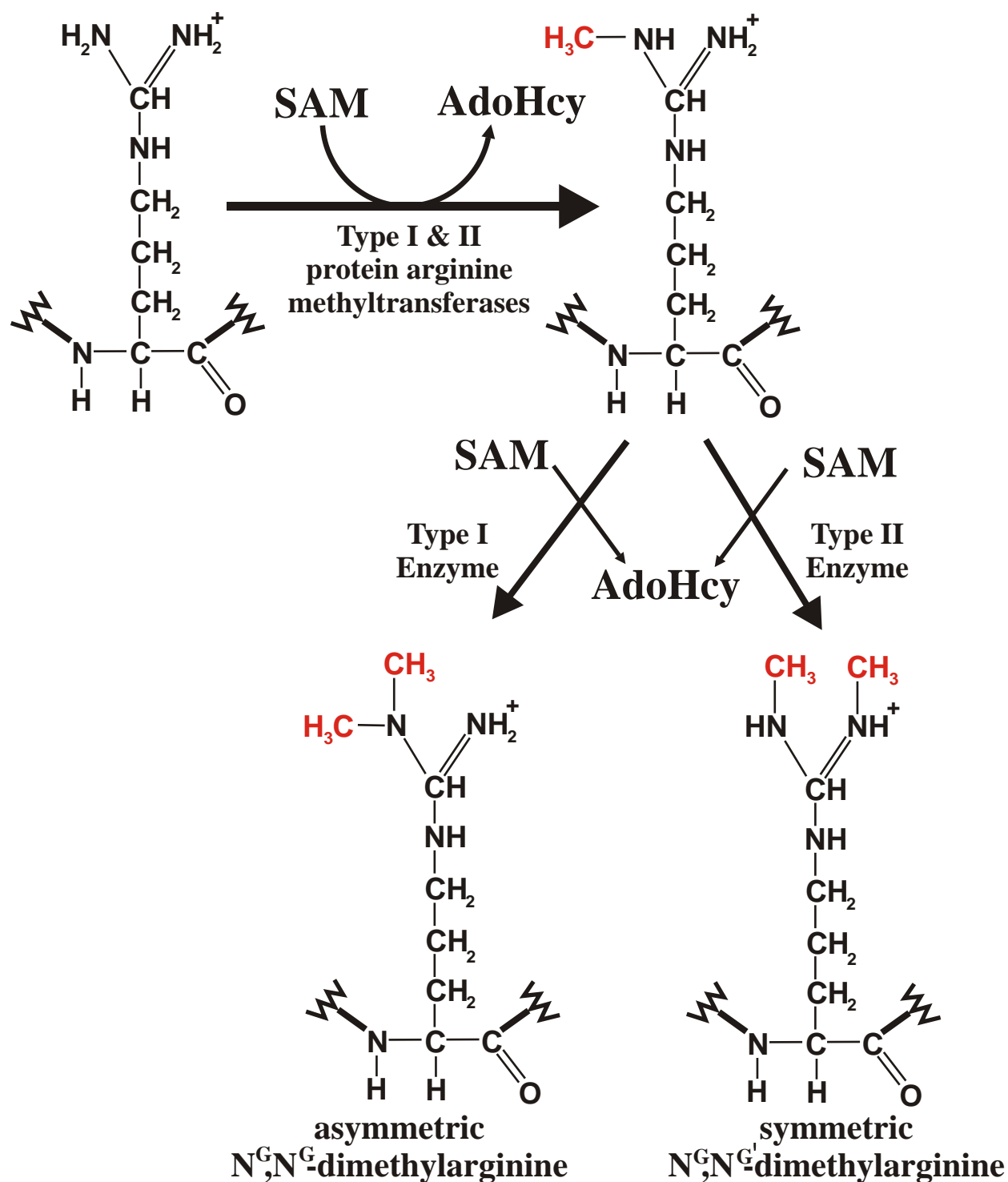
Methylated derivatives of arginine were first identified over 30 years ago as radiolabeled acid hydrolysis products of *in vitro* incubations containing calf thymus nuclei and S-adenosyl-[<sup>14</sup>C-*methyl*]-L-methionine, as well as in studies looking for novel amino acids in urine (Paik and Kim, 1967; Paik and Kim, 1968; Kakimoto and Akazawa, 1970). Until very recently, the enzymes, their substrates, and the functional significance of this post-translational modification were enigmatic and largely unknown. Very recent genetic and molecular biology approaches have rekindled interest in this modification by implicating it in processes from signaling and transcriptional activation to protein sorting (reviewed by Chiang et al., 1996; Kim et al., 1998; Gary and Clarke, 1998; McBride and Silver, 2001).

Three forms of methylarginine have been identified in eukaryotes: N<sup>G</sup>-monomethylarginine (MNA), N<sup>G</sup>,N<sup>G</sup> (symmetric) dimethylarginine (sDMA) and N<sup>G</sup>,N<sup>G</sup> (asymmetric) dimethylarginine (aDMA). All three modifications involve the guanido

nitrogens of the arginine side chain (see Fig. 1.5). S-adenosyl-L-methionine (AdoMet/SAM) is thought to be the universal methyl donor for all enzyme catalyzed methylation events, including arginine methylation (Segal and Eichler, 1989; Chiang et al., 1996). The methyl group from SAM can be transferred to oxygen, nitrogen, sulfur and carbon moieties. A wide variety of both small biological molecules and larger macromolecules can be methylated. They include DNA, RNA, lipids and proteins (Clarke, 1993).

It appears that there are two distinct classes of protein arginine N-methyltransferases, originally defined by substrate specificity. Type I enzymes were originally found to be specific for the histone proteins, and these enzymes catalyze the formation of MNA and aDMA. Type I enzymes were later found to methylate other proteins with a  $K_m$  that is 100 fold better than for the histones. These protein substrates include hnRNP A1, fibrillarin and nucleolin. Type II enzymes were specific for myelin basic protein (MBP), and they catalyze the formation of MNA and sDMA. For both enzymes, symmetric and asymmetric dimethylarginine derivatives pass through the monomethylated form, indicating a stepwise addition of methyl groups.

Classification of Type I and II protein arginine methyltransferases is based on substrate specificity. Type I enzymes cannot methylate MBP, but type II enzymes are capable of methylating Type I substrates, specifically hnRNP A1 and the histones, albeit with a lower  $K_m$  than Type I enzymes. Although the classification is somewhat confusing, Type I and II are generally associated with the final dimethylated product; that is, asymmetric or symmetric DMA, respectively (Gary and Clarke 1998).



**Figure 1.5** The stepwise synthesis of methylated arginine derivatives found in proteins. S-adenosyl-L-methionine (SAM) serves as the methyl donor for two consecutive methylation events (indicated in red) on the guanidine nitrogens of arginine residues. Type I methyltransferases catalyze asymmetric methylation, while Type II methyltransferase catalyze the symmetric methylation.

Purification of Type I enzymes met with variable results. The enzymatic activity of the Type I enzyme is associated with a complex of 275 kDa. This complex is composed of 110 and 75 kDa polypeptides as judged by SDS-PAGE. Several eukaryotic Type I protein arginine methyltransferases (PRMT) exist. Six PRMT's have been cloned in humans, while yeast appears to contain just one, called Hmt1/Rmt1 (Henry and Silver, 1996; Tang et al., 1998). None of the now characterized enzymes encoded by the cloned cDNAs are as large as the smallest polypeptides found in any of the previously purified complexes, raising confusion between early purification attempts and recent cloning results (Henry and Silver, 1996; Lin et al., 1996; Frankel and Clarke, 2000; Tang et al., 2000; Frank et al., 2001; Rho et al., 2001).

On the other hand, the Type II enzymatic activity is associated with a complex of about 500 kDa. This complex is composed of two polypeptides with apparent molecular masses of 100 and 72 kDa. One Type II protein arginine methyltransferase, PRMT5/JBP1, has been cloned with a molecular mass of about 45 kDa, again adding to the confusion between the components with a complex versus activity (Branscombe et al., 2001; Gary and Clarke, 1998).

$N^G, N^G$  dimethylarginine was found to be the major methylated derivative in rat liver nuclei. Surprisingly, nuclear RNA binding proteins contain 65% of the aDMA in the cell (Boffa et al., 1977; Lin et al., 2000). RNA binding proteins shown to contain aDMA, include: the yeast proteins Nsr1, Gar1, Gar2 Hrp1, Nop1, Nab2, Sbp1, Npl3, and the vertebrate proteins Ewing sarcoma protein (EWS), hnRNP A1, hnRNP A2, E1B-AP5, ICP27, Sam68, fibrillarin and nucleolin (Lischwe et al., 1981; Lischwe et al., 1982b; Lischwe et al., 1985; Najbauer et al., 1993; Rajpurohit et al., 1994; Liu and Dreyfuss,



1995; Mears and Rice, 1996; Siebel and Guthrie 1996; Frankel and Clarke, 1999; Valentini et al., 1999; Green et al., 2000; Lin et al., 2000; McBride et al., 2000; Nichols et al., 2000; Bedford et al., 2000; Belyanskaya et al., 2001; Kzhyshkowska et al., 2001). Almost all the proteins that contain asymmetrically dimethylated arginine have GAR domains (see above), and the methylated residues are restricted to these domains. Proteins that contain asymmetrically dimethylated arginine, but not classic GAR domains, are methylated at RXR or RG sequences, with the -1 position almost always a glycine residue (Lischwe et al., 1981; Kim et al., 1997; Hyun et al., 2000; McBride and Silver, 2001). Methylation of arginine has little effect on the charge, or the length and direction of the electric dipole of the guanidinium group. There are, however, subtle changes in the  $pK_a$  of the side chain. The addition of methyl groups increases local hydrophobicity and steric hindrance. Perhaps the most important effect of methylation is the change in hydrogen bonding potential of the arginine side chain due to the removal of amino hydrogens (Raman et al., 2001).

Only recently have we gained a glimpse into the functional significance of arginine methylation. Since arginine methylation is often found in nucleic acid binding proteins, in a domain known to be involved in nucleic acid interactions, it was only logical to examine potential role of protein methylation in nucleic acid binding. However, very limited work has been done in examining this question. The RNA binding of hnRNP A1 to homopolymeric RNA was slightly decreased by methylation, but methylation has no effect on another RNA binding protein, Hrp1. Therefore, the effect of methylation on the ability of a protein to bind RNA remains uncertain (Rajpurohit et al., 1994; Valentini et al., 1999).

The first protein arginine methyltransferase (Hmt1) was cloned by complementation of a synthetically lethal, defective *NPL3* allele. *NPL3* normally encodes a protein involved in poly(A)<sup>+</sup> RNA export (Henry and Silver, 1996). Methylation of Npl3 and another shuttling hnRNP protein, Hrp1, facilitated their export out of the nucleus, thus implicating methylation in the export of mRNA binding proteins. However, another hnRNP shuttling protein, Hrb1, was not affected by methylation (Shen et al., 1998). Therefore, protein methylation might be involved in modulating protein-RNA interactions between mRNA export proteins and their target mRNAs.

Recent studies also implicate methylation in modulating protein-protein interactions. For example, Sam68, a protein thought to act as an adaptor in signaling pathways and a substrate for Src kinase, normally interacts with both WW and SH3 domain-containing proteins. Sam68 shows reduced binding to SH3 proteins when asymmetrically dimethylated, but no change in binding WW domain proteins (Bedford et al., 2000). Similarly, when asymmetrically dimethylated Stat1 (signal transducer and activator of transcription) exhibits decreased affinity for its inhibitor, PIAS1 (Mowen et al., 2001). In contrast, splicing factors Smd1 and Smd3 increase binding to the SMN (survival of motor neuron) protein by 16 fold when symmetrically dimethylated (Brahms et al., 2000; Friesen et al., 2001). All of the above proteins (Sam68, Stat1, Smd1 and Smd3) bind nucleic acids, and arginine methylation appears to affect their binding to protein partners in a nucleic acid independent manners.

Histones were among the first proteins identified as substrates for partially purified Type I protein arginine methyltransferases (Paik and Kim, 1968). Although they contain aDMA, histones are very poor substrates compared to GAR domain containing

proteins, like hnRNP A1, nucleolin and fibrillarin. Methylation of both lysine and arginine in histone tails appears to play roles in epigenetic control of gene expression through involvement in chromatin remodeling (Jenuwein and Allis, 2001). Coactivator-associated arginine methyltransferase 1 / protein arginine methyltransferase 4 (CARM1/PRMT4) was the first identified protein arginine methyltransferase targeted to histones. CARM1 is a transcriptional co-activator of hormone-induced gene activation in rats. The ability to enhance transcription is mediated through the family of p160 coactivators (SRC-1, GRIP1/TIF2, pCIP/RAC3/ACTR/AIB1/TRAM2), specifically by way of its interaction with GRIP1 (Chen et al., 1999). Xu et al. (2001) demonstrated that nuclear hormone-dependent transcriptional activation by CARM1 is via methylation of arginines in histone H3 and CBP/p300 (CREB binding protein). Additionally, CARM1 also acts as a transcriptional repressor of cyclic AMP transcriptional activation mediated by cyclic AMP response element binding protein (CREB). Methylation of specific arginine residues, R580 in p300, and R600 in CBP, block binding to CREB. The methylated arginine found in the KIX domain of CBP/p300 is not in a known methylation motif.

## **GAR DOMAINS**

GAR domains are characterized by the tripeptide motif, RGG. Circular dichroism studies revealed the structure of the GAR domain as an extended  $\beta$ -spiral (Ghisolfi et al., 1992b). The RGG motif is repeated several times within the domain, and the motifs are usually clustered together, interspersed occasionally with an aromatic amino acid such as phenylalanine (see Fig. 1.4). A GAR domain is frequently found in RNA binding proteins including, Gar1, Gar2, Nop1, Nop3, Nab2, Sbp1, Npl3, NopA64, human DNA

helicase II, Ewing sarcoma protein, hnRNP A1, hnRNP A2, and fibrillarin (Russell and Tollervey, 1992; Tollervey et al., 1993; Gulli et al., 1995; de Carcer et al., 1997; Zhang and Grosse, 1997; Frankel and Clarke, 1999; Nichols et al., 2000; Belyanskaya et al., 2001; Green et al., 2002). The GAR domain has been described as a RNA-binding domain, and it does independently exhibit non-specific, low affinity binding to RNA ( $K_d \sim 500$  nm) (Ghisolfi et al., 1992a). Recently, nucleolin's GAR domain was shown to bind G quartets with much higher affinity ( $K_d = 40$  nm) (Hanakah et al., 1999). Both strand annealing and helix destabilizing properties have been attributed to the GAR domain, and these activities may be responsible for the GAR domain's ability to modulate RNA binding by the upstream RBDs in nucleolin (Pontius and Berg, 1990; Kumar and Wilson, 1990; Nadler et al., 1992; Ghisolfi et al., 1992b; Portman and Dreyfuss, 1994; Hanakah et al., 2000). The GAR domain of hnRNP A1 is required for cooperative binding to RNA, but it is also required for interactions with other pre-mRNA binding proteins. Nucleolin's GAR domain shows a specific interaction with ribosomal protein L3, thus strengthening a possible role in protein-protein interactions and perhaps assembly of ribosomal proteins into ribosomes (Cobianchi et al., 1988; Cartegni et al., 1996; Bouvet et al., 1998). The precise functions of nucleolin's GAR domain remain unknown.

This dissertation confirms previous work by others showing that the GAR domain of Chinese hamster nucleolin is required for *in vivo* localization to interphase nucleoli. Examination of *in vivo* protein-protein interactions with other non-ribosomal nucleolar proteins used transiently expressed  $\Delta$ GAR as a novel *in vivo* system. Nopp140 did not redistribute in the presence of nucleoplasmic  $\Delta$ GAR, indicating the Nopp140 and the

amino terminal portion of nucleolin do not interact *in vivo*. Fibrillarin did exhibit a slight increase in its nucleoplasmic signal indicating a possible interaction with  $\Delta$ GAR nucleolin. Transient expression of  $\Delta$ GAR did strongly increase in the nucleoplasmic signal of B23, indicating an interaction with the amino terminal portion of nucleolin and B23.

The objective of this dissertation is to examine the functional associations of the nucleolar protein nucleolin as a window into defining its role in the nucleolus. Additionally, we hope to define a role for the post-translational methylation of the GAR domain of nucleolin, and therefore perhaps provide a model for the methylation of other GAR domain containing RNA-binding proteins.

This dissertation also shows that methylation inhibitors cause nucleolin to redistribute from its normal nucleolar localization, suggesting that methylation is a modulator of macromolecular interactions that are necessary for nucleolin's proper nucleolar localization. The arginines in the glycine arginine rich region of hamster nucleolin were substituted with lysine. The mutant protein was shown not to be a substrate for a protein arginine methyltransferase with broad substrate specificity. This dissertation demonstrates that nucleolin methylation is not required for its proper localization to nucleoli, nor for its nucleic acid binding.

## Chapter 2

### *In Vivo* Protein-Protein Interactions with $\Delta$ GAR Nucleolin

#### INTRODUCTION

Ribosomes are incredibly complex macromolecular assemblies. They are responsible for translating the information stored in our nucleic acids into proteins that then give us form, respond to stimuli, catalyze our reactions, function in transport, provide immunity, regulate gene expression, and ultimately define our existence. For these reasons, ribosomes form a crux of the cell, and therefore a central point of control. Regulation of their existence is of critical importance to the cell; degradation of ribosomes is a steady process related to the age of the ribosome. Therefore, control over ribosome biogenesis must be exerted at the level of their production, and this occurs in the nucleolus. The importance of the nucleolus as a fulcrum of cell regulation is borne out by the number of nucleolar targets affected by central regulators, such as cyclin dependent kinase 1 (CDK1), cyclin dependent kinase 2 (CDK2), retinoblastoma protein (Rb), p53, casein kinase II (CKII), *myc* and CREB binding protein (CBP), all of which have multiple nucleolar targets (Pederson, 1998a; Glover, 1998; Srivastava and Pollard, 1999; Visintin and Amon, 2000; Leary and Huang 2001; Morris and Dyson, 2001; Chan and La Thangue, 2001; Cadwell and Zambetti, 2001; Nigg, 2001). These targets include chromatin remodeling factors, RNA polymerase I transcription factors, and rRNA processing and assembly factors. For these reasons, understanding ribosome biogenesis is of great importance in generating an integrated view of cellular control.

Great effort has been focused on elucidating the mechanisms of ribosome biogenesis, the organization of the ribosomal genes and their transcriptional control, as

well as post-translational modification of the pre-rRNA. Several non-ribosomal nucleolar proteins and small nucleolar RNAs (snoRNAs) are known to be involved in pre-rRNA processing and ribosome assembly. However, the exact functions of the nucleolar proteins remain poorly understood. Pinning down the exact function of these factors has been hampered because ribosome biogenesis is currently impossible to reconstitute *in vitro* due to its complexity. Although lower eukaryotes are amenable to genetic manipulation, the results from such genetic experiments are difficult to interpret because effects are often pleiotropic with factors appearing to have multiple overlapping functions. There are also very distinct differences between vertebrates and yeasts in regards to ribosome biogenesis (see below).

For example, yeast nucleoli are very large compared to nucleoli in vertebrate cells. Yeast nucleoli occupy almost one third of the nucleus, and in the case of *Schizosaccharomyces cerevisiae*, nucleoli do not disassemble during mitosis. The nucleolar sub-compartments of yeast nucleoli are less distinct than the analogous sub-compartments in nucleoli of higher eukaryotes. In fact, until recent advances in cryofixation, these sub-compartments in yeast nucleoli were believed not to exist (Trumtel et al., 2000).

Nucleolar factors involved in ribosome biogenesis vary in their conservation among eukaryotes. For example, NOP1/fibrillarin is highly conserved, with 81% amino acid identity between yeast and vertebrate homologs. Other nucleolar factors, however, like vertebrate nucleolin do not have true homologs in yeast. Gar2p from *Schizosaccharomyces pombe* and Nsr1p from *Schizosaccharomyces cerevisiae* share only 37% identity with vertebrate nucleolin in the most highly conserved domain, the carboxy

terminal GAR domain. The overall amino acid identity between the full-length vertebrate nucleolin and either Gar2p or Nsr1p is much less than 37%. The only real homology between the vertebrate nucleolins and those of yeast is the presence of similar domain structures. In fact, both Gar2p and Nsr1p are considered structural “homologs” of vertebrate nucleolin, but not of each other. Although, Gar1p and Nsr1p share 52% amino acid identity, they are not considered homologs due to distinct differences between their respective domain structures (Aris and Blobel 1991; Kondo and Inouye, 1992; Gulli et al., 1995). There are also several yeast factors; Gar1p1, NOP3, NOP4, NOP5 and NOP77 for which there are no apparent vertebrate homologs (Russell and Tollervey, 1992; Sun and Woolford, 1994; Berges et al., 1994; Bagni and Lapeyre, 1998; Wu et al., 1998). It appears, therefore, that although yeast is a convenient genetic system and a valuable organism that can teach us much about eukaryotic nucleoli, it is not directly applicable to the detailed understanding of vertebrate nucleoli. In order to understand ribosome biogenesis in vertebrates, and how these nucleoli affect vertebrate growth, development, physiology and aging, we must be study vertebrate nucleoli.

Abundant factors implicated in pre-ribosomal processing and/or assembly in vertebrates include Nopp140, fibrillarin, B23 and nucleolin. These nucleolar proteins often appear to have multiple functions. Nucleolin for example, is believed to have roles in ribosomal gene transcription regulation, pre-rRNA folding and processing, import and assembly of ribosomal proteins, maintaining nucleolar structure, and perhaps even signal transduction.

Nopp140 (nucleolar phosphoprotein of 140 kDa), consists almost entirely of alternating acidic and basic domains. The C-terminus is highly conserved, with 59%



identity between rat and yeast. The rest of the protein shows much less conservation. Nopp140 interacts with RPA194 (the largest subunit of RNA polymerase I), and Nopp140 is a component of box C/D and box H/ACA snoRNPs that are respectively involved in 2'-O-methylation and pseudouridine conversion (Miau et al. 1997; Yang et al., 2000). Nopp140 localizes to Cajal bodies and in the DFCs of nucleoli, often in rings around the FCs. This staining pattern may reflect its association with RNA polymerase I and early associating factors involved in the post-translational modification of pre-ribosomal RNA. Similar to several other nucleolar proteins, Nopp140 has both CKII and CDK1 recognition motifs, and it is highly phosphorylated during interphase and mitosis. Nopp140 shuttles between the nucleus and cytoplasm (Meier and Blobel, 1992). Shuttling is faster for Nopp140 than other nucleolar proteins, suggesting a possible role in the transport of nucleolar components (Meier and Blobel, 1992).

The co-localization of nucleolin and Nopp140 in the DFC of nucleoli certainly suggests the possibility for interaction between the two proteins. The alternating acidic and basic domains of Nopp140 could possibly interact with the acidic and basic stretches of amino acids within the amino terminus of nucleolin through electrostatic forces. Nopp140 is known to associate with snoRNPs that interact with the pre-rRNA to act as guides for post-translation modifications. Nucleolin is a tenacious nucleic acid binding protein and has high affinity binding sites in the 5'-ETS of the pre-rRNA primary transcript. Nucleolin might interact with Nopp140 indirectly mediated by ribosomal rRNA. However, to date no interactions between nucleolin and Nopp140 have been observed.

Fibrillarin is a 34 kDa conserved non-ribosomal nucleolar protein (Tollervey et al., 1993). Fibrillarin consists of a well defined amino terminal GAR domain that is rich in aDMA. Two central RNA binding domains (RBDs) follow the GAR domain. Its carboxy domain may form alpha helices (Christensen and Fuxa, 1988; Aris and Blobel, 1991; Lischwe et al., 1985). Like Nopp140, fibrillarin localizes to the DFC, often in a ring surrounding the FC. Fibrillarin is known to be involved in the primary pre-rRNA processing reactions (cleavage) in vertebrates. Fibrillarin is also known to associate with box C/D snoRNAs (methylation) (Tollervey et al., 1993).

Both fibrillarin and nucleolin bind pre-rRNA, localize to the DFC of nucleoli and associate with the U3 snoRNA that is involved in the primary cleavage event in the 5'-ETS of pre-rRNA. Possible interactions between the two proteins might be indirect through pre-rRNA or the U3 snoRNA. The GAR domain of pre-mRNA binding protein, hnRNP A1, was shown to self-associate, indicating that GAR domains might represent protein-protein interaction motifs (Cartegni et al., 1996). It is possible that nucleolin and fibrillarin could interact through their respective GAR domains.

B23 consists of repeating acidic and basic domains, and although it lacks a consensus RBD, B23 can bind nucleic acids. B23 also displays other nucleic acid modulating properties, namely helix destabilization properties and ribonuclease activity (Dumbar et al., 1989; Herrera et al., 1995). The nucleic acid binding properties are associated with its carboxyl terminus. B23 is present in both the DFC and GC (Ochs et al. 1983; Biggiogera et al. 1991). Like nucleolin, B23 shuttles between the nucleolus and cytoplasm (Borer et al., 1989).

Both B23 and nucleolin are among the most abundant nucleolar proteins in actively growing cells, and they are strongly implicated in ribosome biogenesis. Their co-localization in both the DFC and GC, in addition to their apparent central role in ribosome biogenesis, all suggest possible interactions between nucleolin and B23. We already know that B23 interacts with nucleolin by yeast two-hybrid assay and immunoprecipitation experiments (Li et al., 1996), but not as of yet in vertebrate cells.

Previously the GAR domains of some vertebrate nucleolins (*Xenopus*, chicken, rat) were demonstrated to be required for nucleolar localization. Nucleolin lacking this domain accumulated in the nucleoplasm (Heine et al., 1993; Meßmer and Dreyer, 1993; Schmidt-Zachmann and Nigg, 1993). In this chapter, the previous results for  $\Delta$ GAR nucleolin are confirmed for hamster nucleolin, and this abnormal localization is used to examine *in vivo* interactions between  $\Delta$ GAR and the other nucleolar proteins, Nopp140, fibrillarin, and B23.

Nucleolar proteins are dynamic, continuously cycling between the nucleolus and nucleoplasm (Phair and Mistili, 2000). Nucleolar factors that normally interact with the amino terminal two-thirds of nucleolin in the nucleolus should therefore interact with  $\Delta$ GAR in the nucleoplasm, thereby increasing their nucleoplasmic presence. By observing these interactions in non-disrupted living vertebrate cells, we hope to distinguish biologically relevant *in vivo* interactions of nucleolin from those that are not biologically relevant.

Finally, nucleolar proteins are often implicated in diverse cellular functions because immuno-precipitations or similar assays suggest apparent interactions with other various cellular, nuclear and nucleolar protein components. Generation of cell lysates for

immuno-precipitations, however, expose components to conditions that may not exist *in vivo*. It is doubtful that all the interactions observed with nucleolin are biologically relevant, and teasing apart the important facts from the spurious interactions is crucial to understanding the roles of the nucleolar proteins, and therefore, ribosome biogenesis itself.

## **MATERIALS AND METHODS**

### **Construction of Amino Terminal *Myc*-Tagged $\Delta$ GAR**

The Chinese hamster nucleolin cDNA was kindly provided by Dr. Francois Amalric (Centre de Recherche de Biochimie et de Genetique Cellulaires du Centre National de la Recherche Scientifique, France). The cDNA was ligated into the pSP64 vector at the EcoR I and BamH I sites. This construct is 5329 bp in length, and is named pSNH. The cDNA encoding Chinese hamster nucleolin was removed from pSNH and cloned into *myc*-6-D (a derivative of pBluescript KS<sup>+</sup> (Stratagene La Jolla, CA). *Myc*-6-D contains six tandem *myc* tags in the polylinker linker region; it was a gift from Dr. Mark Roth of the Fred Hutchinson Cancer Research Center, Seattle, WA. One of the *myc* tags was removed by cutting *myc*-6-D with Nco I and EcoR I and ligating in the nucleolin cDNA, cut with the same restriction enzymes. In the resulting construct, nucleolin is behind and in frame with the DNA sequence encoding 5 tandem human *myc*-tags such that the nucleolin is tagged at its amino terminus. This construct was created by YingJie Zhu in our lab, and she named it 21R CHO WT. To generate the CHO  $\Delta$ GAR construct, 21R CHO WT was partially digested with Nco I followed by complete digestion with EcoR I. A 5130 bp fragment corresponding to the *myc*-tagged hamster nucleolin cDNA lacking base pairs 1969-2152 (encoding the GAR domain) was gel purified by phenol

and phenol / chloroform extraction. The 5' overhangs left by Nco I and EcoR I were filled in using the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs, Beverly, MA) to generate blunt ends. The filled-in Nco I and EcoR I sites were then ligated together using T4 DNA ligase (New England Biolabs, Beverly, MA) to generate CHO  $\Delta$ GAR. The truncated nucleolin cDNA was subsequently ligated into the eukaryotic expression vector, pBK-CMV (Stratgene, La Jolla, CA) using Xho I and Xba I sites common to both polylinkers. This construct was named CHO pBK-CMV  $\Delta$ GAR.

*Myc*-tagged wild type nucleolin was previously cloned into the pBK-CVM vector in our lab by Dongrai Lu, and this construct was named pMCN. Both pMCN and CHO pBK-CMV  $\Delta$ GAR plasmid DNAs were double CsCl-purified according to Manatis et al. (1982). The ultra pure pMCN and CHO pBK-CMV  $\Delta$ GAR DNA were used to transiently transfect cultured HeLa cells.

### **Transient Transfection of HeLa Cells with $\Delta$ GAR Nucleolin**

HeLa cells were purchased from the School of Veterinary Medicine (Louisiana State University) and grown in DMEM (Debecco's Modified Eagles Medium) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) and 50  $\mu$ g/ml gentamycin (Life Technologies GibcoBRL Gaithersburg, MD) at 37°C in a 5% CO<sub>2</sub> incubator. Actively growing cells were allowed to reach 80-90% confluence in a 75 cm<sup>2</sup> cantered flask before being harvested using 0.25% trypsin (School of Veterinary Medicine, Louisiana State University). Cells were pelleted and resuspended in 5 mls of DMEM with 10% fetal calf serum. Recovered cell densities were determined using a hemocytometer. Coverslips were sterilized by flaming with 95% ethanol and placed in wells of a six well plate. The volume needed to yield  $1.6 \times 10^5$  cells per well was

calculated, and 3.5 mls DMEM with 10% fetal calf serum minus the volume calculated was added to the wells. The calculated volume of cells was then added to the coverslips to bring the total volume of culture medium up to 3.5 mls per well. Cells were then incubated for 24 hours at 37°C in 5% CO<sub>2</sub>, to allow them to adhere to the coverslips. Transient transfections were achieved via DNA-calcium phosphate precipitation using the N<sub>1</sub>N-bis (2-hydroxyethyl)-2- aminoethanesulfonic acid (BES) method of Chen and Okayama (1988). The DNA was incubated with 0.25 M CaCl<sub>2</sub> and 1 x BBS for 15-20 minutes during which time a fine precipitate forms. Ten micrograms of CsCl-purified DNA was added per well. After addition of the DNA precipitate, cells were incubated at 35°C in 3% CO<sub>2</sub> for 12-18 hours to induce uptake of the DNA-calcium precipitate. Cells were then washed twice with 1 x PBS (phosphate buffered saline) and placed in fresh DMEM with 10% fetal calf serum. The cells were subsequently incubated for an additional 24 hours at 37°C in 5% CO<sub>2</sub>,. Cells were then removed from the incubator washed twice in 1 x PBS and fixed for one hour in 2.5% paraformaldehyde in 0.5 x PBS. Cells were blocked with 10% horse serum for 1-24 hours before staining with mouse anti-*myc* mAb 9E10 hybridoma supernatant for one hour, followed by three washes with 10% horse serum. The cells were stained the flourescein-conjugated goat anti-mouse IgG (Pierce, Rockford, IL, USA) diluted 1/200 in 10% horse serum.

### **Construction and Integration of C-terminally EGFP-tagged Chinese Hamster $\Delta$ GAR Nucleolin**

The GAR domain was deleted from nucleolin in pSNH using the internal Nco I site located at nucleotide +1886 and the EcoR I from the downstream polylinker region of the pSP64 vector. The 5' overhanging ends were filled-in using *Taq* DNA polymerase (Promega Corp., Madison, WI) at 72°C, for 20 minutes. Two hundred units of T4 DNA

ligase (New England Biolabs, Beverly MA) were used to blunt-end ligate the resulting ends of the plasmid to generate pSNH  $\Delta$ GAR. Sequence encoding nucleolin  $\Delta$ GAR was cut out of pSNH  $\Delta$ GAR using Xba I (upstream polylinker) and EcoR I (downstream polylinker). The resulting 1886 bp fragment was gel purified and ligated into pEGFP-N3 (Clontech LaJolla, CA) at the Xba I and EcoR I sites in the vector maintaining the reading frame with the carboxyl terminal EGFP peptide. The same procedure was used to construct a plasmid that encodes full-length wild type hamster nucleolin from the pSNH vector. The resulting vectors, named pEGFP-N3 CHO ( $\Delta$ GAR or WT) were sequenced at the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA) (<http://www.dna.iastate.edu/>) to confirm that the nucleolin cDNA was in frame with the sequences encoding EGFP peptide. Ultra pure plasmid DNA, either pEGFP-N3 CHO  $\Delta$ GAR or pEGFP-N3 CHO WT DNA, was prepared via double CsCl gradient ultracentrifugation according to Manatis et al. (1982).

Chinese hamster ovary (CHO) cells were purchased from the School of Veterinary Medicine (Louisiana State University) and grown to 80% confluency in 125 mm<sup>2</sup> sterile tissue culture petri dishes. When growing the CHO cells, the DMEM was supplemented with 1  $\mu$ M proline from a frozen sterile 100  $\mu$ M stock. CHO cells were transfected via DNA-calcium phosphate precipitation using the N<sub>1</sub>N-bis (2-hydroxyethyl) - 2 - aminoethanesulfonic acid (BES) method of Chen and Okayama (1988). Twenty micrograms of CsCl-purified pEGFP-N3 CHO  $\Delta$ GAR or pEGFP-N3 CHO WT DNA were incubated with 0.25 M CaCl<sub>2</sub> and 1x BBS for 15-20 minutes. After addition of the DNA precipitate to the cells, the cells were incubated at 35°C in 3% CO<sub>2</sub> for 16 hours. The cells were then washed twice with 1 x PBS and placed in fresh DMEM with 10%

fetal calf serum and incubated for 48 hours at 37°C in 5% CO<sub>2</sub> to allow strong expression of the neomycin resistance gene. G418 (Life Technologies GibcoBRL, Gaithersburg, MD) was then added to the media to a final concentration of 1 µM after 48 hours. The cells were allowed to grow under strong selection of G418 (1µM) until colonies appeared, usually between three to four weeks during which time the media was replaced every three days.

To establish clonal purity, cells were treated with 0.25% trypsin to remove them from the petri dish, washed once in 1 x PBS and DMEM before determining cell concentration using a hemocytometer. Cells were then diluted with DMEM to a concentration of 15 cells / ml and 20 µl of this dilution was added to each well of a 96 well plate. Each well already contained 300 µl of DMEM with 10% fetal calf serum and 1 µM G418. This dilution and plating should yield an average of one cell every three wells. Culture medium was replaced every four days for the first two weeks. Wells containing a single visible colony were scraped with a 1 ml pipette to detach some of the cells that were then placed in a six well plate containing ethanol-sterilized coverslips and 3 mls of DMEM with 10% fetal calf serum and 1 µM G418. Cells were incubated for up to four days, washed twice in 1 x PBS, and fixed in 2.5% paraformaldehyde in 0.5 x PBS for one hour. The cells were visualized by fluorescence microscopy to determine which colony displayed consistent high-level expression of EGFP-tagged nucleolin. Those cells were then collected from either the original 96 well plate and/or the 6 well subculture plate via scraping. These cells were placed in a 75 cm<sup>2</sup> tissue culture flask with DMEM with 10% FCS and grown to 80% confluency in the presence of G418 before using them



in an experiment or freezing them in liquid nitrogen. The freezing medium consisted of 90% fetal calf serum and 10% DMSO.

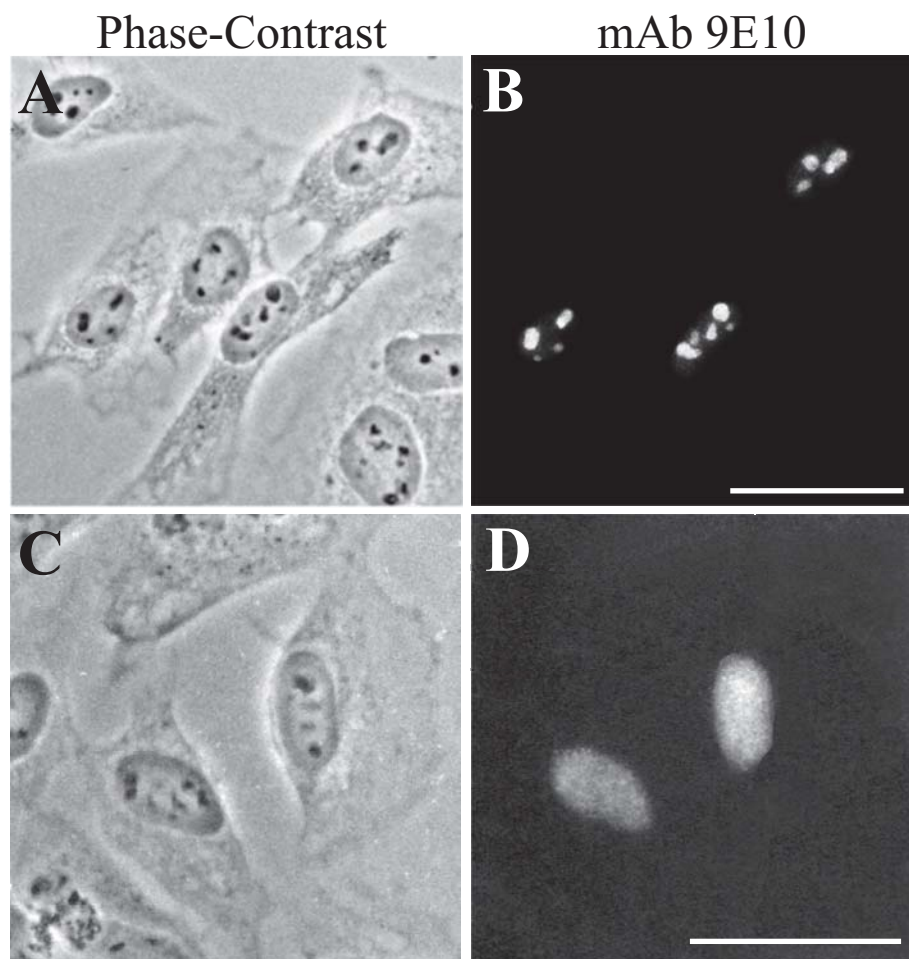
### **Potential *In Vivo* Protein-Protein Interactions of Nucleolin with Other Nucleolar Proteins**

CHO cells that stably expressed C-terminal EGFP-tagged wild type or  $\Delta$ GAR nucleolin were stained with antibodies directed at other nucleolar proteins to determine if the nucleoplasmic  $\Delta$ GAR could increase the nucleoplasmic signal of these other nucleolar proteins. Cells that stably expressed C-terminal EGFP-tagged wild type or  $\Delta$ GAR nucleolin were harvested using 0.25% trypsin, pelleted and resuspended in DMEM with 10% fetal calf serum. Recovered cell densities were determined using a hemocytometer. Coverslips were sterilized by flaming with 95% ethanol and placed in a six well plate. The volume needed to yield  $1.6 \times 10^5$  cells per well was calculated and 3.5 mls DMEM with 10% fetal calf serum minus the volume calculated was added to the wells. The calculated volume of cells was then added to the coverslips to bring the total volume of DMEM with 10% fetal calf serum up to 3.5 mls per well. Cells were incubated for 24 hours at 37°C in 5% CO<sub>2</sub> to allow them to adhere to the coverslips. Attached cells were washed twice in 1 x PBS and fixed in 2.5% paraformaldehyde in 0.5 x PBS for one hour, prior to being permeabilized with 0.5% Triton X-100 in 1 x PBS for five minutes. Cellular proteins were then blocked with 10% horse serum for 1-3 hours before staining with primary antibody. The primary antibodies were mAb 72B9 directed against fibrillarin (Reimer et al., 1987) (1/100), anti-B23 antibodies courtesy of Dr. Ben Valdez directed against B23 (1/400), and mAb RH10 courtesy of Dr. Tom Meier directed against Nopp140 (1/100). Cells were washed and then reblocked with 10% horse serum prior to staining with the appropriate secondary antibody that was coupled to rhodamine.

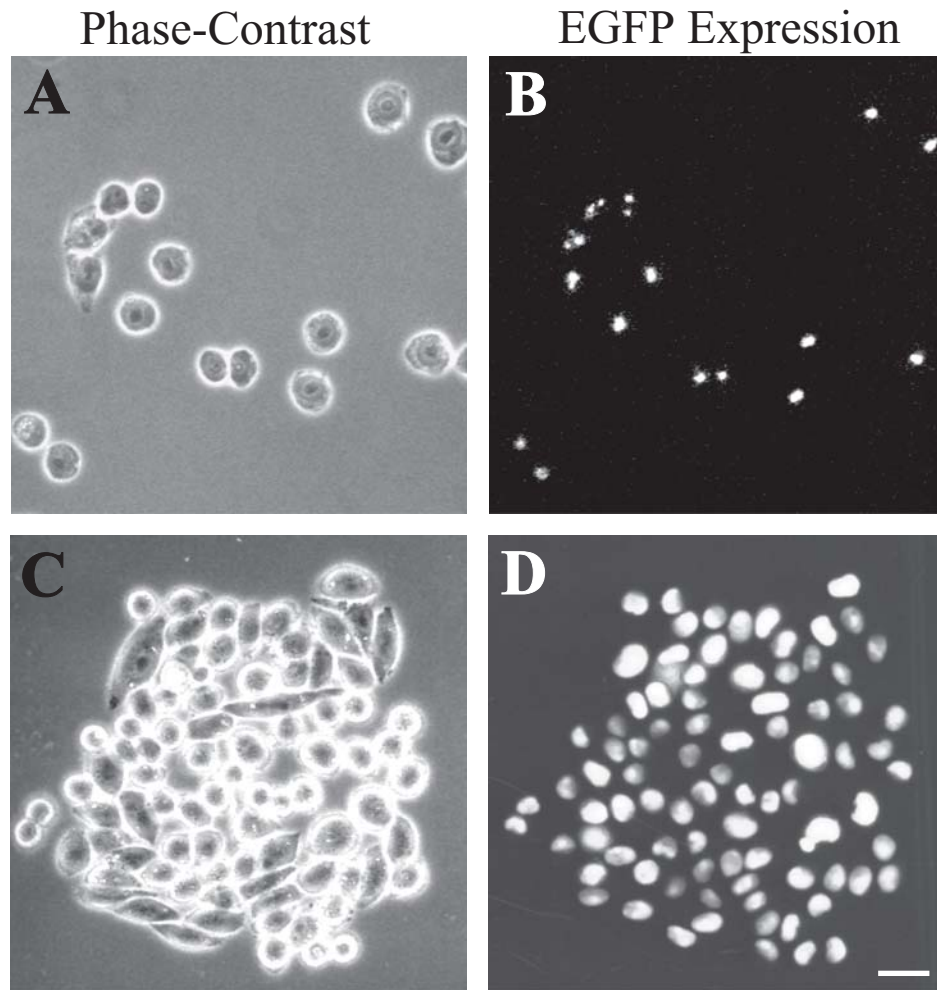
Standard phase contrast and immuno-flourescence microscopy was preformed using a Ziess Axioskop using a 40x phase contrast/neoflour objective. Pictures were taken with Fuji Etkachrome 400 speed color slide film or Kodak T-Max 400 speed black and white film.

## RESULTS

Expression of *myc*-tagged Chinese hamster nucleolin lacking its GAR domain ( $\Delta$ GAR) via transient transfection in HeLa cells demonstrated that the GAR domain of hamster nucleolin was required for *in vivo* localization to interphase nucleoli. Nucleolin lacking its GAR domain failed to localize to interphase nucleoli, but instead exhibited uniform staining of the nucleoplasm (see Fig. 2.1, C and D). This was not the case for the wild type *myc*-tagged full-length nucleolin (see Fig. 2.1, A and B). The results also indicate that the amino terminal *myc*-tag tag did not interfere with nucleolar localization. For convenience, the enhanced green fluorescent protein (EGFP) was fused to the carboxyl terminus of Chinese hamster nucleolin lacking its GAR domain by ligating the truncated nucleolin cDNA into the eukaryotic expression vector, pEGFP-N3, behind the strong CMV promoter. A similar construct with wild type CHO nucleolin was created to act as a control in subsequent experiments. The plasmids containing the fusion proteins were transfected into CHO cells and selected for stable intergration using G418. High level expression could be obtained initially for both the wild type and GAR truncated fusion proteins (see Fig 2.2). However, there was a continuous loss of expressing cells over time even under continued selection. The loss of expression was particularly acute for the  $\Delta$ GAR nucleolin fusion protein with roughly 30% reduction in the number of expressing cells after each passage of a 25 cm<sup>2</sup> flask. After four passages in the presence



**Figure 2.1** HeLa cells transfected with wild type *myc*-tagged nucleolin (panels **A** and **B**) or *myc*-tagged nucleolin lacking the GAR domain ( $\Delta$ GAR) (panels **C** and **D**). Cells were stained with the monoclonal antibody 9E10 followed by goat anti-mouse flourescein-conjugated secondary antibody. Calibration bars are 20  $\mu$ m for all panels.



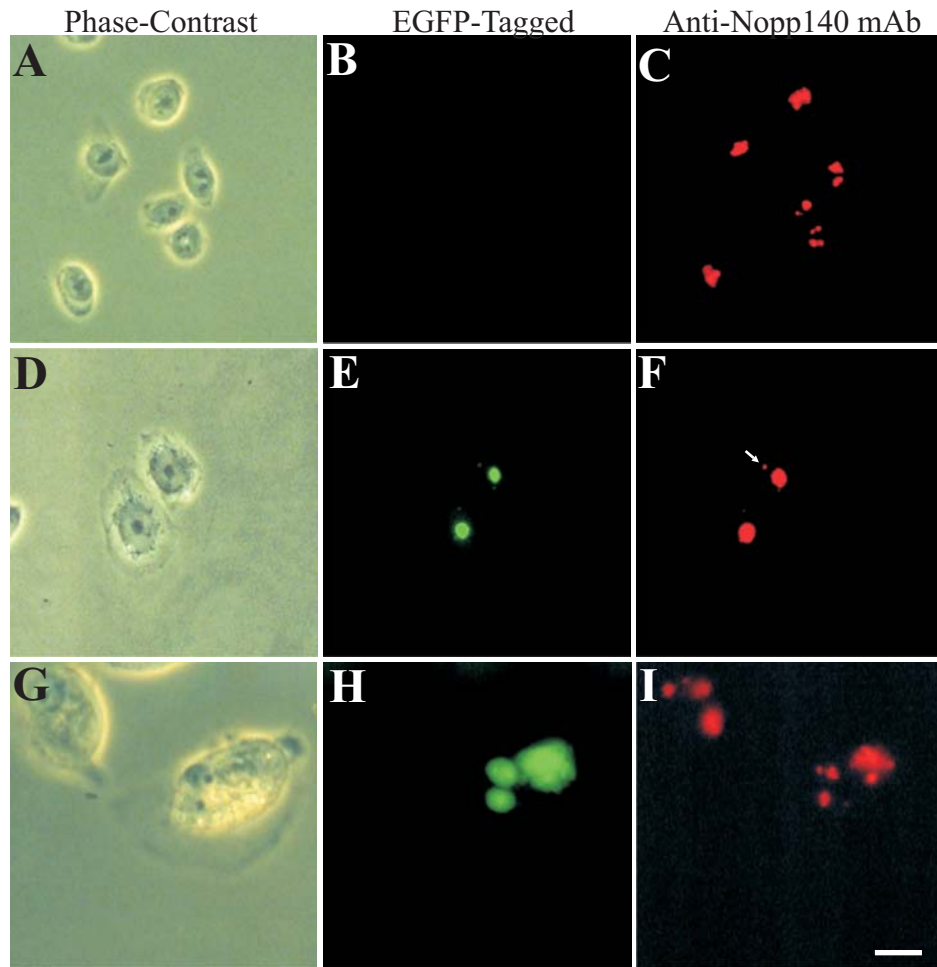
**Figure 2.2** CHO cells stably expressing GFP-tagged wild type and  $\Delta$ GAR nucleolin. Cells pictured after selection in G418 for a total of three weeks. Panels A and B show CHO cells stably expressing wild type nucleolin that is C-terminally tagged with EGFP. Panels C and D show CHO cells stably expressing  $\Delta$ GAR nucleolin that is C-terminally tagged with EGFP. Calibration bar is 20  $\mu$ m for all panels.

of continued selection (approximately four weeks), there was complete loss of cells expressing the  $\Delta$ GAR fusion protein.

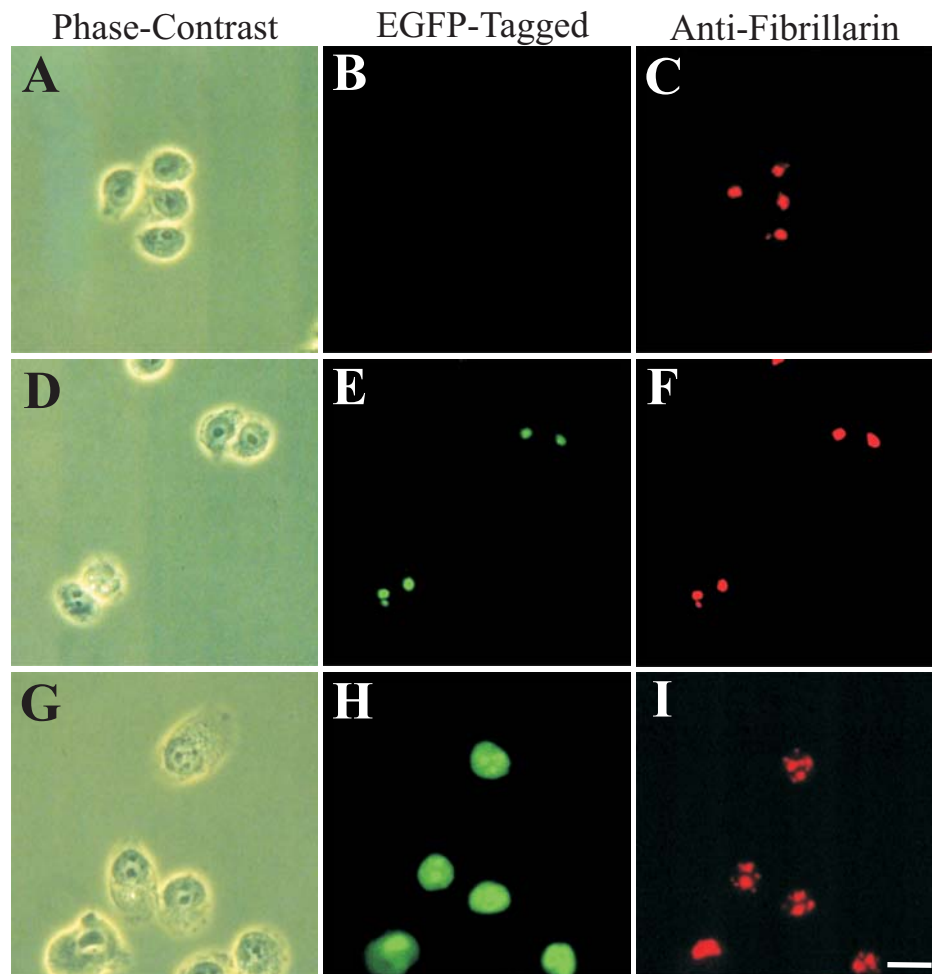
The wild type nucleolin-EGFP fusion protein fared much better with only a 10-15% decrease in the number of cells expressing the fusion protein after each passage in a 25 cm<sup>2</sup> flask. Some cells continued to express the wild type fusion protein after four months of culture (~12 passages).

The expression of the fusion proteins slightly altered the morphology of the nucleoli in the CHO cells. Nucleoli in cells expressing the full-length version of nucleolin were larger than untransfected controls (see Figs. 2.2, 2.3, 2.4, A and C). This result is consistent with the finding of Zhu et al. (1999) who showed that over-expression of nucleolin in CHO cells caused enlarged DFCs. The nucleoli in cells expressing nucleolin lacking its GAR domain were occasionally slightly smaller than nucleoli in control cells (see Fig. 2.2D and C), suggesting some loss of nucleolar material, perhaps to the nucleoplasm due to an interaction with the nucleoplasmic-localized  $\Delta$ GAR nucleolin (see below).

The EGFP-tagged  $\Delta$ GAR nucleolin does not localize to interphase nucleoli. Instead, it is evenly distributed throughout the nucleoplasm. Since nucleolar proteins are dynamic, continuously cycling between the nucleolus and nucleoplasm, this would potentially allow other nucleolar proteins that normally interact with the amino terminal two-thirds of nucleolin in the nucleolus to interact with  $\Delta$ GAR in the nucleoplasm. Such a dynamic shuttling and interaction with nucleolin would increase their respective nucleoplasmic signals (Phair and Mistili, 2000). Antibodies to three abundant nucleolar



**Figure 2.3** Localization of endogenous Nopp140 in Chinese hamster ovary cells. Panels A-C are untransfected CHO cells as a control of anti-Nopp140 antibody staining. Panels D-F are CHO cells stably expressing EGFP-tagged wild type nucleolin showing co-localization of EGFP-nucleolin and endogenous Nopp140. Panels G-H are CHO cells stably expressing EGFP-tagged  $\Delta$ GAR nucleolin, with no increase in the nucleoplasmic signal of Nopp140 due to  $\Delta$ GAR nucleolin. Calibration bar is 20  $\mu$ m for all panels.



**Figure 2.4** Localization of endogenous fibrillarin in Chinese hamster ovary cells. Panels A-C show untransfected CHO cells as a control of anti-fibrillarin staining. Panels D-F show CHO cells stably expressing EGFP-tagged wild type nucleolin that co-localizes with endogenous fibrillarin. Panels G-H show CHO cells stably expressing EGFP-tagged  $\Delta$ GAR nucleolin, with a slight increase in the nucleoplasmic signal of fibrillarin due to  $\Delta$ GAR nucleolin. Calibration bar is 20  $\mu$ m for all panels.

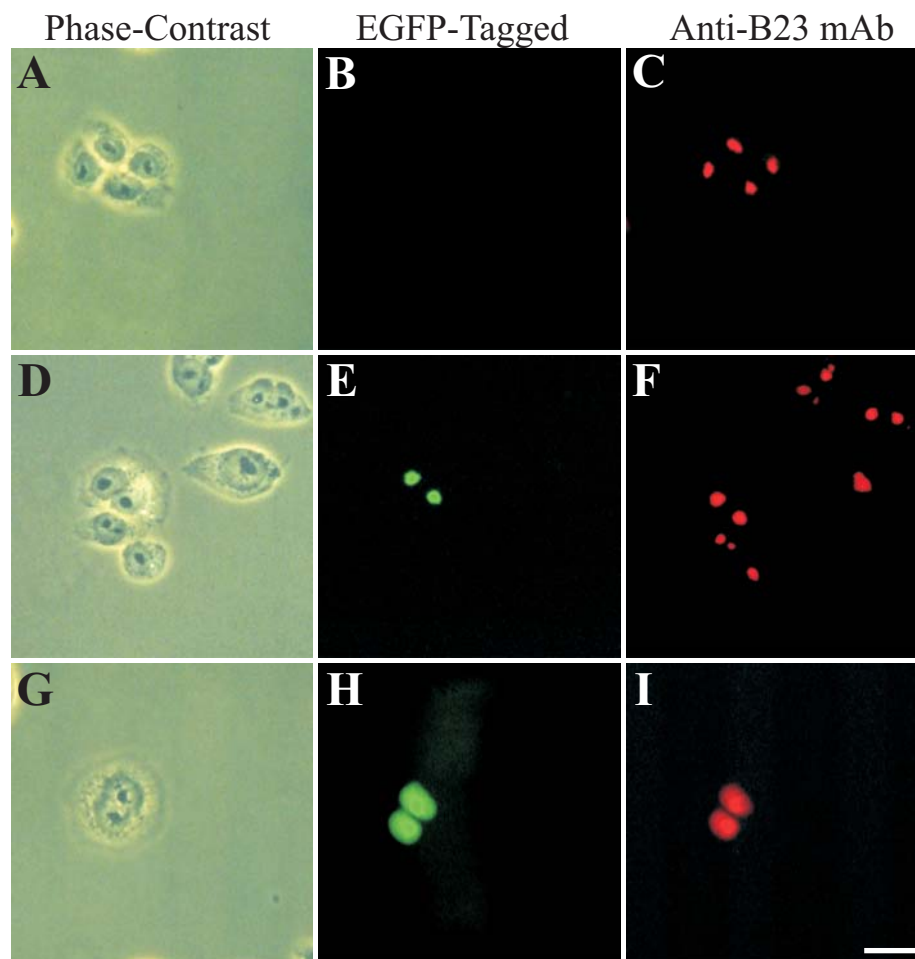
proteins (Nopp140, fibrillarin and B23) were used as probes to examine their localization in the presence of wild type or  $\Delta$ GAR EGFP-tagged nucleolin in transfected CHO cells.

Endogenous Nopp140 localizes to nucleoli in untransfected CHO cells (see Fig. 2.3, A and C). This localization is undisturbed in the presence of EGFP-tagged wild type nucleolin (see Fig. 2.3, D-F). In panel F of Figure 2.3, the arrow indicates Nopp140 localizing to a Cajal body, indicating that expression of the EGFP-tagged construct does not interfere with the behavior or localization of Nopp140. In cells expressing  $\Delta$ GAR nucleolin, Nopp140 is unaffected in its intra-nuclear distribution, exhibiting no increase in its nucleoplasmic signal. These normal localizations suggest no direct or indirect interaction with the amino terminal two thirds of nucleolin and endogenous Nopp140.

Endogenous fibrillarin localizes to nucleoli in untransfected CHO cells (see Fig. 2.4, A and C). In the presence of  $\Delta$ GAR nucleolin, the amount of fibrillarin in the nucleoplasm, as detected by indirect immunostaining, appeared to increase slightly (see Fig. 2.4, H and I), possibly indicating a weak or indirect interaction with the amino terminal two-thirds of nucleolin. However, this result was somewhat variable from cell to cell. The expression of EGFP-tagged full-length nucleolin did not alter the staining pattern of endogenous fibrillarin (see Fig 2.4, E and F), thus indicating that the increased nucleoplasmic signal in the presence of  $\Delta$ GAR is not simply due to the over expression of the  $\Delta$ GAR EGFP-tagged construct.

Endogenous B23 localizes to nucleoli in untransfected CHO cells (see Fig. 2.5, A and C). In the presence of exogenously expressed EGFP  $\Delta$ GAR nucleolin, however, there was a dramatic increase in B23 in the nucleoplasm as indicated by indirect-





**Figure 2.5** Localization of endogenous B23 in the Chinese hamster ovary cells. Panels A-C are untransfected CHO cells as a control of anti-B23 staining. Panels D-F are CHO cells stably expressing EGFP-tagged wild type nucleolin showing co-localization of EGFP-nucleolin and endogenous B23. Panels G-H are CHO cells stably expressing EGFP-tagged  $\Delta$ GAR nucleolin, with a distinct increase in the nucleoplasmic signal of B23 due to  $\Delta$ GAR nucleolin. Calibration bar is 20  $\mu$ m for all panels.

immunostaining (see Fig. 2.5, H and I). Since B23 localizes normally to nucleoli in the presence of full-length EGFP-tagged nucleolin (see Fig. 2.5, E and F), the redistribution seen in the presence of  $\Delta$ GAR is not simply due to the over expression of the EGFP-tagged construct. The increase in the nucleoplasmic signal of B23 in the presence of exogenously expressed  $\Delta$ GAR EGFP nucleolin suggests an *in vivo* interaction between B23 with the amino terminal two-thirds of nucleolin. Previous work supports this observation (Li et al., 1996). The results therefore suggest that  $\Delta$ GAR has limited or no interaction with Nopp140, a slight interaction with fibrillarin, and a significant interaction with B23 when stably expressed in CHO cells.

## **DISCUSSION**

### **Expression of $\Delta$ GAR Nucleolin in HeLa Cells**

Our lab and others previously showed that the GAR domains of various vertebrate nucleolins were required for nucleolar localization, and that nucleolin lacking this GAR domain accumulated in the nucleoplasm (Heine et al., 1993; Meßmer and Dreyer, 1993; Schmidt-Zachmann and Nigg, 1993). Chinese hamster nucleolin lacking its GAR domain failed to localize to interphase HeLa cell nucleoli (see Fig. 2.1, C and D). The results presented here agree with results from other organisms, namely that the GAR domain of nucleolin is required for proper localization of nucleolin to nucleoli.

The nucleolar localization of nucleolar proteins is presumed to be the result of functional associations during ribosome biogenesis. Specifically, the localization of nucleolin to nucleoli is believed to be mediated by binding pre-rRNA; RNase treatment induces the release of nucleolin from nucleoli (Schwab and Dreyer, 1997). The failure of

$\Delta$ GAR to localize to nucleoli is possibly related to its inability to efficiently bind nucleic acids (see Chapter 4) (Heine et al., 1993).

### **Possible Interactions with Other Non-Ribosomal Nucleolar Proteins in CHO Cells**

We noticed that cells expressing  $\Delta$ GAR appeared to have smaller nucleoli than non-transfected cells (see Figs. 2.2, 2.3, 2.4, A and C). This might be due to  $\Delta$ GAR nucleolin interacting with other nucleolar components, and thus causing them to relocate from the nucleolus to the nucleoplasm, thereby decreasing the size of the nucleolus. Enhanced green fluorescent protein (EGFP) was fused to the carboxy terminus hamster nucleolin (wild type and  $\Delta$ GAR) in order to leave nucleolin's amino terminus unobstructed. The amino terminal portion of nucleolin has been shown to be involved in interactions with histones, snoRNP U3, and some ribosomal proteins (Erard et al., 1988; Ginisty et al., 1998; Bouvet et al., 1998).

CHO cells were transfected with constructs expressing either wild type or  $\Delta$ GAR EGFP-tagged Chinese hamster nucleolin. Stable integrations of the plasmids expressing the EGFP tagged constructs were selected by G418 resistance. Cells expressing EGFP-tagged nucleolins, however, could not be perpetuated indefinitely in cell culture before losing expression of EGFP as monitored by fluorescence microscopy. Loss of wild type expression was slow (about 10% of the cells per week). This is most likely the result of cytotoxicity of the EGFP tag, which has been demonstrated for stable expression from strong promoters in cell culture (Liu et al. 1999; Gubin et al. 1999). The loss of expression of  $\Delta$ GAR nucleolin, however, was more acute with a decline rate of 4-5 times that of wild type. This suggests that something other than GFP toxicity was interfering with the cells ability to grow and / or maintain expression of EGFP  $\Delta$ GAR nucleolin.

This loss of viability is likely due to interference with ribosome biogenesis as a result of  $\Delta$ GAR nucleolin sequestering components needed for ribosome biogenesis in the nucleoplasm.

In order to test the above hypothesis, antibodies to three other nucleolar proteins were used on cells expressing  $\Delta$ GAR EGFP nucleolin. The three nucleolar proteins examined were Nopp140, fibrillarin, and B23. The amount of nucleoplasmic staining of the three nucleolar proteins in CHO cells expressing  $\Delta$ GAR EGFP nucleolin was compared to staining in untransfected cells or in cells expressing wild type EGFP nucleolin. Nopp140 appeared to be unaffected by the presence of  $\Delta$ GAR nucleolin. Fibrillarin was more difficult to characterize because although there did appear to be a slight redistribution of fibrillarin in the presence of  $\Delta$ GAR nucleolin, the redistribution was weak and inconsistent (see Fig. 2.4). B23 on the other hand, clearly redistributed to the nucleoplasm in the presence of  $\Delta$ GAR (see Figs. 2.3 and 2.5 respectively).

Nopp140 is a nucleolar phosphoprotein of 140 kDa. It consists almost entirely of alternating acidic and basic domains. Nopp140 normally localizes to the DFC of the nucleolus and to Cajal bodies. Similar to other nucleolar proteins, Nopp140 shuttles between the nucleus and cytoplasm (Meier and Blobel, 1992). This shuttling, together with the ability of Nopp140 to bind NLSs, suggests a possible role of Nopp140 in the transport of nucleolar components (Meier and Blobel, 1992). Nopp140 is also a component of box C/D and box H/ACA snoRNPs, and it may have a role in the transport of snoRNPs to the nucleolus (Yang et al., 2000). Although the alternating acidic and basic stretches common to both nucleolin and Nopp140 provide potential interaction surfaces, no interaction has been demonstrated for these two proteins. Our results also

did not indicate an interaction between Nopp140 and  $\Delta$ GAR *in vivo*;  $\Delta$ GAR nucleolin failed to redistribute Nopp140 to the nucleoplasm (see Fig. 2.3, H-I).

Fibrillarin is a highly conserved nucleolar protein. It consists of an amino terminal GAR domain, two central RNA binding domains (RBDs), and a carboxy terminal domain that may form alpha helices (Christensen and Fuxa, 1988; Aris and Blobel, 1991). Similar to Nopp140, fibrillarin is associated with box C/D snoRNAs, and it localizes to the DFC of nucleoli (Tollervey et al., 1993). In yeast, fibrillarin is implicated in maturation of all rRNAs species, which strongly indicates a role for fibrillarin in ribosome biogenesis.

In the presence of  $\Delta$ GAR nucleolin, fibrillarin appears to be slightly redistributed to the nucleoplasm (Fig. 2.4, H-I). The redistribution of fibrillarin in the presence of  $\Delta$ GAR was, however, weak and inconsistent from cell to cell. Fibrillarin is known to be associated with the U3 snoRNP in mouse, and nucleolin's amino terminus has been shown to directly interact with U3 snoRNA in mouse (Turley et al., 1993; Ginisty et al., 1998). Although no direct interaction between fibrillarin and nucleolin has been observed previously, the slight redistribution of fibrillarin by  $\Delta$ GAR may be the result of an indirect interaction mediated by the U3 small nucleolar RNA (snoRNA).

B23 is a multifunctional nucleolar protein present in both the DFCs and GCs (Ochs et al. 1983; Biggiogera et al. 1991). Similar to other nucleolar proteins (Nopp140 and nucleolin) the amino terminus of B23 consists of repeating acidic and basic domains. Although B23 lacks a consensus RNA binding domain, it can bind nucleic acids, presumably through its carboxyl terminus (Dumbar et al., 1989). B23 shuttles between the nucleolus and cytoplasm, and it can bind monopartite SV40-type NLSs, but not the

classic bipartite NLSs. This binding has been demonstrated to be responsible for directing viral proteins Rev, Rex and possibly Tat to the nucleolus (Borer et al., 1989; Fankhauser et al., 1991; Szebeni et al., 1995). Non-ribosomal nucleolar proteins, nucleolin and p120 have been shown to interact with B23 (Li et al., 1996; Valdez et al., 1994). B23 is considered a putative ribosome biogenesis factor based on its localization and nucleic acids binding.

Previous work demonstrated an interaction between nucleolin and B23 by a yeast two-hybrid assay and immuno-precipitation (Li et al., 1996). Deletion analysis identified amino acids 194-239 of human B23 as critical for its interaction with nucleolin. This region includes part of the ill-defined, C-terminal region that is responsible for the nucleic acid binding ability of B23 (Li et al., 1996; Wang et al., 1994). Deletion analysis identified amino acids 540-628 of human nucleolin as critical for the interaction with B23. This region comprises consensus sequence RNA binding domains 3 and 4. The methods used to demonstrate the interaction between nucleolin and B23 by Li et al. (1996) did not exclude the possibility of the interaction being mediated by nucleic acids. Furthermore, the domains defined as essential for the interaction between the two proteins are both involved in nucleic acid binding.

B23 was clearly redistributed to the nucleoplasm in the presence of EGFP  $\Delta$ GAR (see Fig. 2.5, H-I), but not by the presence of wild type EGFP-tagged nucleolin. Thus over-expression of the EGFP-tagged wild type nucleolin did not affect endogenous B23. RBDs 3 and 4, defined as critical for interaction with B23, are present in  $\Delta$ GAR nucleolin, and therefore one would expect to see an interaction with B23 based on the previous data of Li et al. (1996). The interaction of B23 and nucleolin as reported by Li

et al. (1996) did not directly address the requirement for the presence of the GAR domain. Additionally, the interaction seen by Li et al. (1996) could have been mediated by nucleic acids. Our current effort agrees with the work of Li et al. (1996) and extends it by suggesting an *in vivo* interaction between nucleolin and B23 in a vertebrate cell. Since the GAR domain of nucleolin is needed for efficient nucleic acid binding, the *in vivo* interaction seen here suggests that the interaction between B23 and nucleolin is not mediated by nucleic acids.

## Chapter 3

### Effect of Methylation Inhibition on Chinese Hamster Nucleolin *In Vivo*

#### INTRODUCTION

Post-translational modification of proteins allows cells to expand beyond the limits imposed by the twenty encoded amino acids. Just as protein phosphorylation has proved to have important implications in protein function, other post-translational modifications, such as arginine methylation, may represent a powerful modulator of protein function. Despite methyl arginines being discovered over 30 years ago, only very recently has any functional significance been ascribed to this post-translational modification. Arginines destined for methylation are typically located within specific motifs depending on the type of methylated end product generated. Three types of methylated arginines are generated *in vivo*, monomethylarginine, symmetric dimethylarginine and asymmetric dimethylarginine (see Fig. 1.5). Symmetric dimethylarginines are located in RG dipeptide repeats or RXR motifs. Asymmetric dimethylarginines are located in RGG motifs that are often clustered together with interspersed aromatic amino acids (usually phenylalanine) in a protein domain known as the glycine and arginine rich (GAR) domain (Lischwe et al., 1981; McBride and Silver, 2001).

The recent discoveries regarding the functional significance of arginine methylation have been mainly for the symmetrically dimethylated form. These discoveries include protein-protein interactions related to cell signaling, transcription and protein sorting (McBride and Silver, 2001; Friesen et al., 2001). Myelin basic protein (MBP), a major component of the myelin sheath, was the first protein known to be



symmetrically dimethylated. Only one arginine appears to be methylated corresponding to residue 107 of the bovine sequence. MBP is located in compact myelin and interacts with opposed cytoplasmic membrane surfaces. Symmetric dimethylation may play an essential role in maintaining of the layered membrane of the myelin sheath (Gary and Clarke, 1998).

Very recently other proteins were shown to contain sDMA. These include splicing factors SmD1, SmD3 and signaling proteins Stat1 and Sam68. For these proteins, methylation appears to be involved in protein-protein interactions. In the case of SmD1 and SmD3, symmetric dimethylation increases binding to the SMN protein. Alternatively, binding to target proteins PIAS1 and SH3 domain-containing proteins by Stat1 and Sam68, respectively, decreased upon symmetric dimethylation.

Most methylated arginines, however, are asymmetrically dimethylated. To date, no clear functional significance is known for asymmetrically dimethylated proteins involved in ribosome biogenesis (i.e. nucleolin and fibrillarin which are among the most highly methylated cellular proteins). Histones were among the first proteins identified as substrates for partially purified Type I protein arginine methyltransferases (Paik and Kim, 1968). It is now known that although they contain aDMA, histones are very poor substrates compared to GAR domain-containing proteins like hnRNP A1, nucleolin and fibrillarin.

Both lysine and arginine methylation of histone tails appears to play roles in the epigenetic control of gene expression through involvement in the “histone code” mediated chromatin remodeling (Jenuwein and Allis, 2001). Recent cloning of several mammalian protein arginine methyltransferases has allowed the identification of

previously unknown substrates for methylation. Coactivator-associated arginine methyltransferase 1 / protein arginine methyltransferase 4 (CARM1/PRMT4) was identified as a transcriptional co-activator of hormone-induced gene activation. The ability to enhance transcription is mediated through the family of p160 coactivators (SRC-1, GRIP1/TIF2, pCIP/RAC3/ACTR/AIB1/TRAM2), specifically by way of its interaction with GRIP1 (Chen et al., 1999). Xu et al. (2001) demonstrated that nuclear hormone-dependent transcriptional activation by CARM1 is via methylation of arginines in histone H3 and CBP/p300 (CREB binding protein). Additionally, CARM1 acts as a transcriptional repressor of cyclic AMP transcriptional activation mediated by cyclic AMP response element binding protein (CREB). Methylation of specific arginine residues in p300 (R580) and in CBP (R600) blocks binding of CREB. The methylated arginine, however, does not exist in a known methylation motif. This is, however, the first detailed mechanism for asymmetric dimethylation function, and it represents the first function of aDMA in vertebrates (Xu et al., 2001). Other than the role of asymmetric methylation of arginine in CARM1 in transcriptional control, the functional significance of asymmetric dimethylation in vertebrates is generally unknown for aDMA containing-RNA binding proteins.

Methylation of yeast shuttling hnRNP proteins Npl3 and Hrp1 facilitates their export from the nucleus. Both proteins bind poly A<sup>+</sup> RNA, and blocking their methylation dependent nuclear export is correlated with a nuclear accumulation of poly A<sup>+</sup> RNA. These results implicate protein methylation as having a role in nucleocytoplasmic shuttling (Gary et al., 1996; Shen et al., 1998).

High molecular weight fibroblast growth factor-2 (HMW FGF-2) (an alternative 22/24 kDa form of the protein produced by translation from an alternative CUG start codon) is localized to the nucleus as opposed to the normal 18 kDa form which is cytoplasmic. The high molecular weight form of FGF-2 has five to eight arginines depending on the particular form produced. These arginines are located in classic RGG motifs in an internal GAR domain and are known to be asymmetrically dimethylated. Deletion of the GAR domain or treatment with methyltransferase inhibitors, 5-deoxy 5' methylthioadenosine (MTA) or 3-deaza-adenosine (DAA), causes HMW FGF-2 to shift its intracellular steady state concentration from the nucleus to the cytoplasm (Pintucci et al., 1996; Klein et al., 2000). The localization of another aDMA containing protein, hnRNP A2, is also shifted from the nucleus to the cytoplasm upon deletion of its GAR domain, or upon treating cells with methyltransferase inhibitors (Nichols et al., 2000). Methylation is therefore thought to be responsible for the nuclear localization of the protein.

The previous chapter demonstrated that the GAR domain of Chinese hamster nucleolin is required for proper localization of nucleolin to interphase nucleoli, indicating that the GAR domain is involved in targeting nucleolin to nucleoli. Since the GAR domain is involved in localization and aDMAs are located solely within this domain, asymmetric dimethylation may be involved in protein targeting. Additionally, previous results had demonstrated a potential link between methylation and the cellular localization HMW FGF-2 and hnRNP A2, thus reinforcing this idea. This chapter describes the relocation of nucleolin from nucleoli to the nucleoplasm in the presence

of the same methyltransferase inhibitors. The observation supports the hypothesis that nucleolin methylation regulates its intracellular localization.

## **MATERIALS AND METHODS**

### **MTA and DAA Inhibition of Methylation**

HeLa cells were grown on coverslips in six well culture plates at 37° C in a 5% CO<sub>2</sub> atmosphere. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Gibco BLR, Gaithersburg, MD) supplemented with 10% fetal calf serum and 50 µg/ml gentamycin. HeLa cells were transfected with the pBK-CMV expression vector encoding a *myc*-tagged wild type hamster nucleolin (pMCN). Transfection was by the calcium phosphate method of Chen and Okayama (1988) using N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid. After adding the Ca<sup>2+</sup>-DNA solution, cells were cultured at 35°C in a 3% CO<sub>2</sub> atmosphere for 18 hours. The calcium-DNA precipitate was washed off with sterile 1 x PBS, and the cells allowed to express tagged nucleolin for 24 hours in DMEM with 10% FCS at 37° C in 5% CO<sub>2</sub> prior to treatment with methyltransferase inhibitors.

Methyltransferase inhibitors MTA and DAA were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Cells were placed in medium containing 3 mM MTA or 30 µM DAA for up to three hours at 37° C and in a 5% CO<sub>2</sub> atmosphere. Some cells were fixed and stained immediately upon removal of MTA, while others were allowed to recover in fresh medium for up to two hours. Some HeLa cells were also placed in medium containing 100 µg/ml cycloheximide for three hours alone, or for two hours in the presence of cycloheximide prior to treatment with MTA for one hour. Cells were fixed in 2.5% paraformaldehyde in 0.5 x PBS for 2 hours at room temperature, followed by three

washes in 1 x PBS. Cells were made permeable with 0.5% Triton X-100 in 1 x PBS for 5 minutes, and preblocked with 10% horse serum in 1 x PBS at room temperature for 1.5 hours. Cells were stained with anti-*myc* mouse monoclonal antibody 9E10 hybridoma supernatant for 1 hour at room temperature followed by washing and restaining with fluorescein-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) for 1 hour at room temperature. A Zeiss Axioskop was used to visualize cells by standard phase contrast and immuno-flourescence microscopy. Pictures were taken with Kodiak T-Max<sup>®</sup> 400 speed black and white film.

### **Conformation of Inhibition of Protein Synthesis by Cycloheximide**

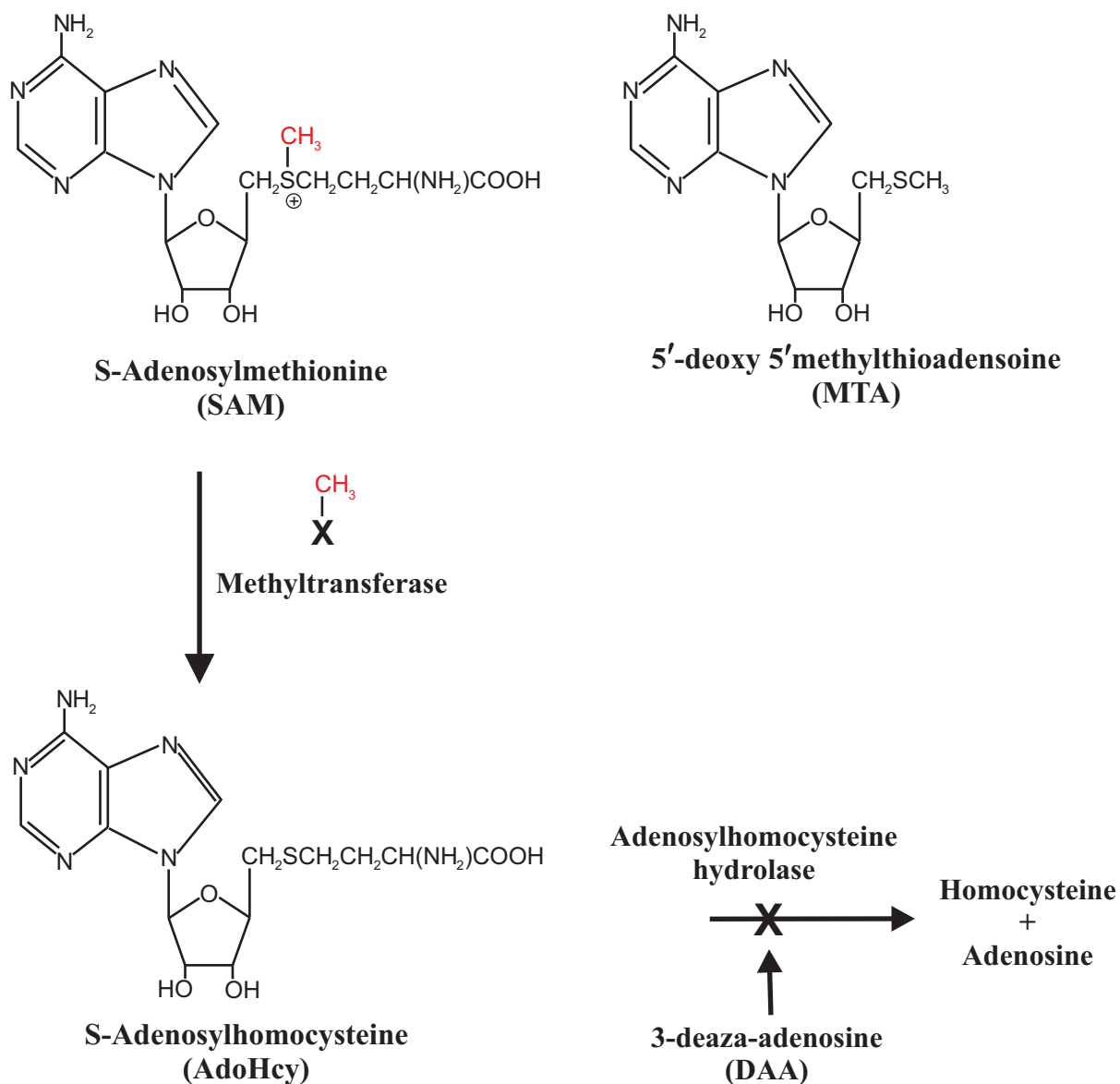
HeLa cells were cultured as described above, except that no coverslips were included in the wells, and the seeding density was increased to  $9.4 \times 10^5$  cells/well. Three wells were treated with 100 µg/ml cycloheximide for one hour prior to incubation of all six wells with 50 µl of [<sup>14</sup>C] labeled amino acid mix (Gly, Ala, Pro, Val, Leu, Iso, Thr, Glu, His, Arg, specific activity of 1 µCi/ul) in 1 x PBS for 20 minutes. To remove the cells from the wells, they were treated with 0.25% trypsin in 1 x PBS either with or without 100 µg/ml cycloheximide. The cells were washed twice in 1 x PBS. They were then resuspended in 50 µl of 0.1% Triton X-100 and vortexed vigorously followed by homogenization with 50 strokes of a disposable dounce homogenizer in a 1.5 ml Eppendorf tube. The entire sample was then applied to a Whatman GF-A glass micro-fiber filter and left to air dry for 25 minutes. The filters were placed in ice-cold 10% TCA for one minute, followed by treatment with hot (~99°C) 10% TCA for 10 minutes to break the amino acyl-tRNA bond. Filters were then rinsed in 100% ethanol followed by

ether under gentle suction. Each filter was placed in 10 mls of scintillation fluid and assayed using a LS 6000IC Beckman (Model # LSBK-1C) scintillation counter.

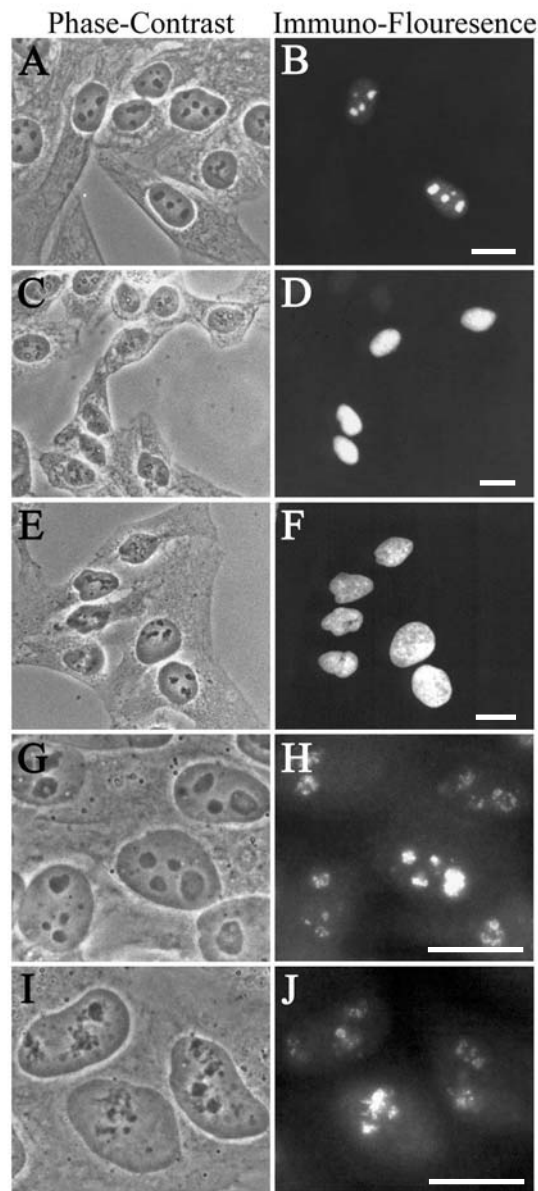
## RESULTS

### Effect of Methyltransferase Inhibitors MTA and DAA on Nucleolin in HeLa Cells

Chapter 2 described how the GAR domain of Chinese hamster nucleolin is necessary for nucleolar localization. The GAR domain contains several conserved dimethylarginines, the functions of which remain unknown. Work by Pintucci et al. (1996), Klein et al., (2000) and Nichols et al. (2000) indicated arginine methylation affects intracellular distribution of HMW-FGF2 and hnRNP A2. A review by Ginisty et al. (1999) of nucleolin also suggested that methylation might play a role in its localization to nucleoli. To explore this possibility, HeLa cells were transfected with plasmids encoding *myc*-tagged versions of wild type nucleolin. Methylation of the transfected cells was blocked using MTA and DAA, known protein methyltransferase inhibitors (see Fig 3.1) (Pintucci et al., 1986). After as little as one hour in culture medium containing 3 mM MTA, the *myc*-tagged nucleolin had redistributed to the nucleoplasm, and nucleoli in most cells appeared fragmented (see Fig. 3.2, and 3.3, C and D). Redistribution of nucleolin to the nucleoplasm was also seen in cells treated with DAA. The effect of DAA, however, was slower than MTA with some nucleolin redistribution seen in two hours and strong redistribution observed after 18 hrs (see Fig 3.2, D and E). The remnant nucleoli in cells in which nucleolin had redistributed to the nucleoplasm exhibited detectable amounts of fibrillarin as determined by antibody staining (see Fig 3.2, I and J). This indicates that not all aDMA containing nucleolar proteins relocalize as a result of treatment of cells with these methylation inhibitors.

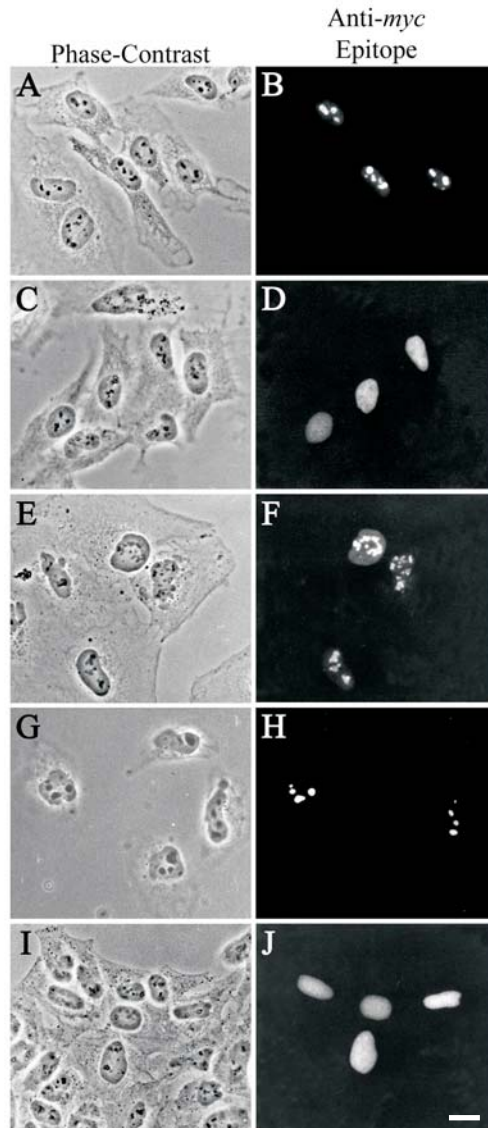


**Figure 3.1** Structure of S-adenosylmethionine (SAM), 5'-deoxy 5'-methylthioadenosine (MTA), and S-adenosylhomocysteine (AdoHcy). SAM donates the methyl group indicated in red to X which represents a methyl acceptor. The methyltransferase inhibitor, MTA, is an analog of SAM. The methyltransferase inhibitor DAA acts to inhibit the enzyme adenosylhomocysteine hydrolase, thus preventing the breakdown of AdoHcy into homocysteine and adenosine. The increasing concentration of AdoHcy then acts to inhibit the methyltransferase.



**Figure 3.2** Effects of blocking SAM-dependent methylation using the methyltransferase inhibitors, MTA and DAA. HeLa cells were transfected with a cDNA encoding *myc*-tagged full-length wild-type hamster nucleolin (**A-F**). *Myc*-tagged nucleolin properly localizes to HeLa cell interphase nucleoli (**A** and **B**). After treatment for 2 hours with 3mM MTA in the culture medium, *myc*-tagged nucleolin redistributes to the nucleoplasm (**C** and **D**). The same redistribution is seen after 18 hours of treatment with DAA (**E** and **F**). Endogenous fibrillarin localizes to nucleoli in untreated cells (**G** and **H**). Fibrillarin's nucleolar localization is unchanged by treatment with 3mM MTA for two hours (**I** and **J**). Calibration bar is 20  $\mu$ m for all panels.





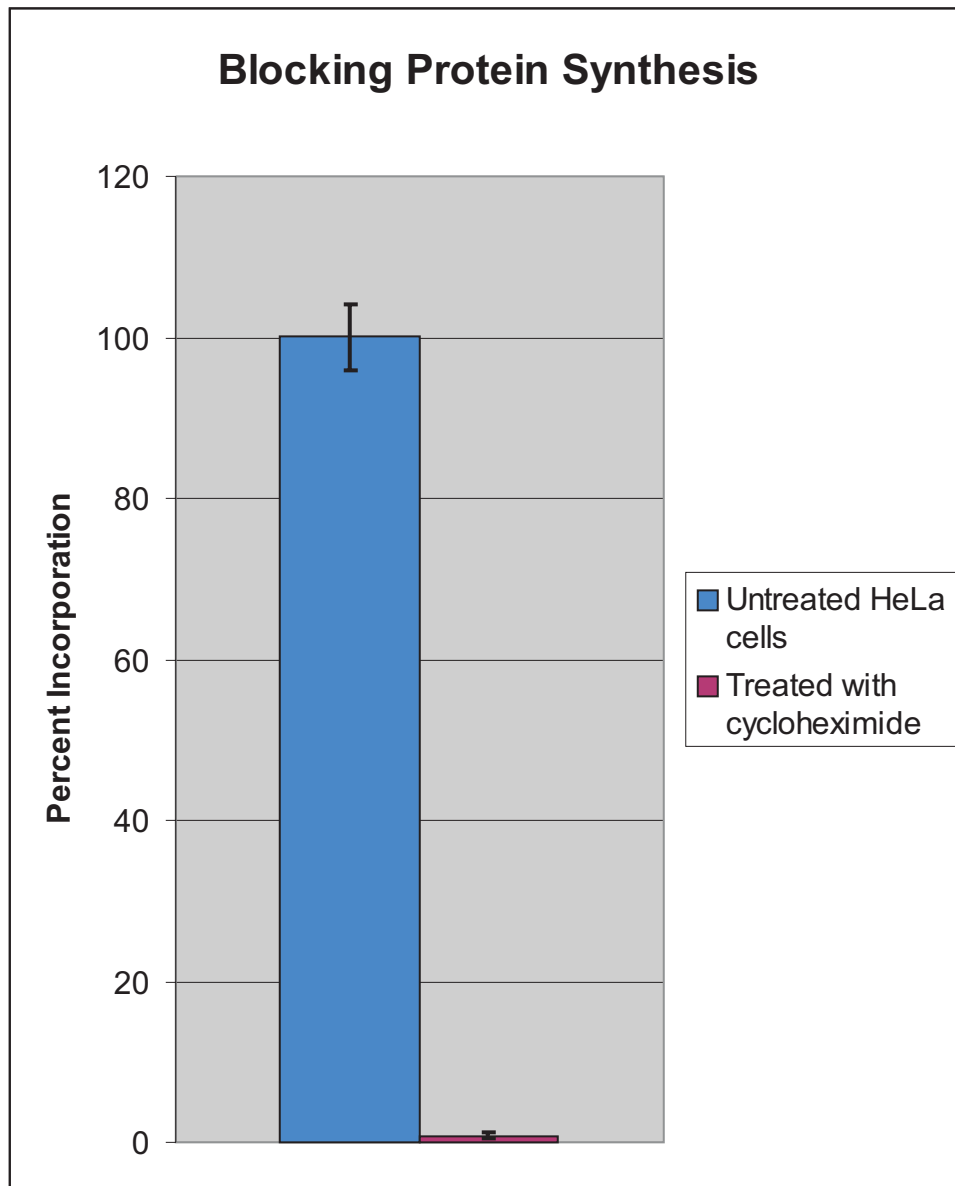
**Figure 3.3** *Myc*-tagged nucleolin properly localizes to nucleoli in transfected interphase HeLa cells (**A** and **B**). Treatment with 3 mM MTA for 2 hours caused the *myc*-tagged nucleolin to redistribute to the nucleoplasm (**C** and **D**). Within one hour after removal of MTA from the culture medium, *myc*-tagged nucleolin re-associated with interphase nucleoli (**E** and **F**). Addition of 100 µg/ml cycloheximide to the culture medium had no effect on the distribution of *myc*-tagged nucleolin after three hours (**G** and **H**). Pre-treatment of HeLa cells with 100 µg/ml cycloheximide for 2 hours to block protein translation had no effect on the MTA-mediated redistribution of *myc*-tagged nucleolin (**I** and **J**). Bar is 20 µm for all panels.

Since the mode of action was more direct and the effect more rapid with MTA than with DAA, MTA was used in all subsequent experiments. When treated cells were allowed to recover in fresh medium lacking MTA, *myc*-tagged nucleolin reassociated with nucleoli in as little as 30 minutes, and all cells showed nucleolin reassociation within two hours (see Fig. 3.3, E and F).

The apparent redistribution of nucleolin after MTA treatment could have been due to the affinity of newly synthesized and unmethylated *myc*-tagged nucleolin to enter the nucleus by not the nucleolus. To test this possibility, we used cycloheximide to block translation prior to treatment with MTA. Pretreatment of HeLa cells with 100 µg/ml cycloheximide for one hour was determined to be a sufficient block for all protein translation (see Fig 3.4). This concentration of cycloheximide had no effect on nucleolin's association with nucleoli (see Fig. 3.3, G and H). When HeLa cells were treated with MTA after pretreatment with cycloheximide, *myc*-tagged nucleolin was again observed to redistribute to the nucleoplasm (see Fig.3.3, I and J). This indicates that the nucleoplasmic signal was not due to nascent protein entering the nucleus, but instead a redistribution of nucleolin that had been associated with nucleoli prior to the addition of MTA to the culture medium. The result is consistent with methylation inhibitors causing the redistribution of aDMA containing proteins.

## **DISCUSSION**

Both methyltransferase inhibitors used in this study, MTA and DAA, demonstrated a pronounced effect on the behavior of exogenously expressed *myc*-tagged hamster nucleolin. After only one hour of MTA treatment, exogenous nucleolin had redistributed to the nucleoplasm. In some cells the nucleolar remnants appeared as dark



**Figure 3.4** Graph showing the inhibition of protein synthesis by cycloheximide. HeLa cells were either treated with 100  $\mu\text{g/ml}$  cycloheximide for one hour or left untreated. The amount of  $[^{14}\text{C}]$  labeled amino acids are represented as a percent incorporation compared to untreated HeLa cells. Protein synthesis is almost completely blocked, with treated cells incorporated only 0.8% as much radiolabel  $[^{14}\text{C}]$  as untreated cells.

holes in the nucleoplasm indicating a complete lack of nucleolin (see Fig. 3.3, C and D). MTA also affected the morphology of the interphase HeLa cell nucleoli, causing them to become fragmented. This effect may be directly due to the loss of nucleolin and B23.

DAA was much slower acting than MTA with 12-16 hours being required for complete redistribution of the exogenous nucleolin. The difference in the behavior of the two methyltransferase inhibitors probably reflects their different modes of action. MTA is a structural homolog of S-adenosyl-L-methionine (SAM), the universal methyl donor. MTA, therefore, most likely inhibits methylation by directly competing with SAM in binding the active site of protein methyltransferases. DAA, on the other hand, inhibits methylation indirectly by blocking adenosylhomocysteine hydrolase, the enzyme responsible for converting S-adenosylhomocysteine into adenosine and homocysteine (see Fig 3.1). Excess S-adenosylhomocysteine exerts considerable end-product inhibition on SAM dependent enzymes (Chiang, 1996). The delayed effect of DAA is most likely due to the lag time required to increase intracellular concentrations of adenosylhomocysteine to levels capable of inhibiting the methyltransferases. These results agree with experiments on other aDMA containing-proteins, HMW FGF2 and hnRNP A2, in which a block in their methylation adversely affects their intracellular distribution (Pintucci et al. 1996; Nichols et al., 2000).

Not all aDMA containing nucleolar proteins are affected by treatment with the methyltransferase inhibitor, MTA. The localization of endogenous fibrillarin appears unaffected by treatment with MTA (see Fig. 3.2, I and J). This is somewhat surprising since fibrillarin contains a GAR domain that is highly methylated *in vivo* (Christensen and Fuxa, 1988). The GAR domain of fibrillarin is near the amino terminus of the

protein, and the domain is positioned much more internally compared to the GAR domain of nucleolin. Unlike the GAR domain of nucleolin, the GAR domain of fibrillarin has never been shown to be required for nucleic acid binding or nucleolar localization. The differences in the behavior of nucleolin and fibrillarin in the presence of MTA may reflect distinct functional differences between their respective GAR domains.

The redistribution of exogenous nucleolin by MTA suggests that arginine methylation may be involved in maintaining the nucleolar localization of nucleolin. Redistribution of exogenous nucleolin due to treatment with MTA occurs quickly, taking approximately one hour. When MTA is washed out, nucleolin returns to interphase nucleoli in approximately the same one hour time period required for MTA to redistribute nucleolin to the nucleoplasm. If nucleolin's nucleolar localization is due to the state of its arginine methylation, then one of two possibilities exists. In the first possibility, the protein arginine methyltransferase responsible for nucleolin methylation is nuclear, and this activity must be maintained to keep nucleolin localized to nucleoli. Alternatively, the loss of nucleolin localization from nucleoli may be the result of nascent unmethylated protein entering the nucleus and being unable to localize to nucleoli without the presence of aDMAs in the GAR domain. The second scenario would require that the cellular turnover of nucleolin be very rapid.

In order to distinguish between these two possibilities, we determined conditions to block protein synthesis (see Fig 3.4). In the presence of cycloheximide, exogenous nucleolin behaved identically in the presence of MTA, redistributing to the nucleoplasm (see Fig 3.3, I and J). This result indicates that the nucleolin that redistributed to the nucleoplasm was originally localized to nucleoli prior to the addition of the MTA. Thus,

the staining of the nucleoplasm is not the result of unmethylated nascent nucleolin entering the nucleus and being unable to localize to nucleoli. Assuming that the redistribution of nucleolin in the presence of MTA is directly related to blocking arginine methylation in the GAR domain of nucleolin, the nucleolar localization of nucleolin is therefore dependent on arginine methylation.

If the methylation of nucleolin is required for its nucleolar localization and blocking methylation results in redistribution of nucleolin to the nucleoplasm, then nucleolin must be in a cycle of methylation and demethylation *in vivo*. The shuttling of nucleolin is far slower than the kinetics of nucleolar reassociation observed after removal of MTA from the medium. Therefore, the protein arginine methyltransferase responsible for nucleolin methylation would need to be nuclear in order to meet the kinetics of redistribution and relocalization if arginine methylation is controlling nucleolin localization. The drawback in these assumptions is the belief that protein methylation is thought to be irreversible (Clarke, 1998).

Protein arginine methyltransferase 1 (PRMT1) is responsible for 90% of type I protein arginine methyltransferase activity in vertebrate cells (Tang et al., 2000b). PRMT1 is localized primarily to the nucleus with small amounts detected in the cytoplasm (Tang et al., 1998). It is not known if nucleolin is an *in vivo* substrate for PRMT1; however, PRMT1 does exhibit strong methyltransferase activity toward RGG motifs *in vitro*. The nuclear localization of the PRMT1 does concur with the hypothesis that nucleolin may be demethylated, and that the unmethylated form of nucleolin is unable to associate with the interphase nucleoli. There is, however, no evidence of an

enzymatic activity related to demethylation, or that post-translationally methylated proteins can be demethylated other than by proteolysis.

MTA must block global methylation in the cell. This includes DNA methylation, RNA methylation and various forms of protein methylation. Therefore, the effects that MTA has on nucleolin localization may not be directly related to nucleolin's methylation state, but rather MTA's effects may be indirect due to lack of methylation of other nucleolar substrates. For example, another nucleolar substrate could be pre-rRNA. The 2'-O-methylation of pre-rRNA that is mediated by box C/D snoRNPs and fibrillarin should also be inhibited by MTA, since this methylation is a SAM-dependent event (Chiang et al., 1996). Nucleolin's interaction with pre-rRNA may be directly dependent on the methylation state of pre-rRNA, either indirectly through a mediator of ribose methylation or directly through structural changes induced in the pre-rRNA by methylation of sugars. Fibrillarin is known to be involved with the 2'-O-ribose methylation of pre-rRNA. Perhaps fibrillarin, which is not redistributed upon treatment with MTA, remains associated with pre-rRNA until methylation is complete. Alternatively, nucleolin may bind the pre-rRNA only once the RNA is methylated.

Blocking methylation of pre-rRNA may also prevent the further production of pre-rRNA by a feedback mechanism involving RNA pol I. When transcription of rRNA is inhibited by actinomycin D, nucleoli disassemble and nucleolin redistributes to the nucleoplasm, indicating the need for active transcription to maintain association of nucleolin nucleolar morphology (Escande et al., 1985; DiMario unpublished results). The disruption of nucleolar morphology and the redistribution of nucleolin in the presence of MTA could be the result of MTA blocking the methylation of pre-rRNA and

therefore its maturation. A block in pre-rRNA maturation may then feedback to stop further transcription of polymerase I. There are probably a number of additional ways that blocking methylation may produce the observed effects, and for this reason an experiment that directly addresses the effect of nucleolin's arginine methylation is presented in Chapter 4.



## Chapter 4

### Characterization of an Arg→Lys Substituted Nucleolin

#### INTRODUCTION

The research performed in Chapter 3 suggests arginine methylation is important for the sub-cellular localization of nucleolin, and therefore, its function. The redistribution of nucleolin, however, might not be directly related to the effects of blocking nucleolin methylation with MTA or DAA. Instead a block in the methylation of other nucleolar components may cause nucleolin to redistribute. Also, due to relatively large intracellular pools of SAM, we were unable to biochemically correlate the effects of MTA with the state of nucleolin methylation *in vivo*. In this chapter I describe attempts to directly test the supposition that arginine methylation plays a role in the localization of nucleolin to nucleoli as initially proposed by Ginisty et al. (1998).

Dimethylarginine exists as two distinct forms *in vivo*, symmetric and asymmetric (see Fig. 1.5). Symmetric arginines are located in RG dipeptide repeats or RXR motifs. Asymmetric arginines are typically located in RGG motifs that are often clustered together with interspersed aromatic amino acids in a protein domain known as the glycine arginine rich (GAR) domain (McBride and Silver, 2001; Lischwe et al., 1981).

The functional significance of symmetric arginine methylation includes protein-protein interactions, transcription and protein sorting (McBride and Silver, 2001; Friesen et al., 2001). Symmetric methylation of myelin basic protein (MBP) may play an essential role in maintaining the correct membrane-associated position of MBP (Gary and Clarke, 1998). The protein-protein interactions involving splicing factors SmD1 and SmD3, as well as signaling proteins Stat1 and Sam68, appear to be modulated by

symmetric arginine dimethylation (McBride and Silver, 2001). For example, symmetric dimethylation of SmD1 and SmD3 increases binding to the survival of motor neuron (SMN) protein. Alternatively, binding to target proteins PIAS1 and SH3 domain-containing proteins by Stat1 and Sam68 respectively, decreased upon symmetric dimethylation of Stat1 and Sam68.

The function of asymmetric methylation is known in only one case. When the KIX domain of the CBP/p300 (CREB binding protein) is asymmetrically dimethylated, it is no longer able to interact effectively with the KID domain in the cyclic AMP response element binding protein (CREB). Thus, the interaction between CBP/p300 and CREB is disrupted. CBP/p300, however, is very atypical for aDMA-containing proteins. Its arginine is not located in a classic RGG motif, and it is the substrate for a very specific protein arginine methyltransferase, CARM1/PRMT4 (Xu et al., 2001). All other known aDMA-containing proteins are methylated in RGG motifs that are typically found in a GAR domain. Proteins containing RGG motifs are substrates for the major cellular protein arginine methyltransferase, PRMT1, which is responsible for 90% of the type I protein arginine methyltransferase activity in the cell (Tang et al., 2000a; Tang et al., 2000b). GAR domain containing proteins are also among the most highly methylated cellular proteins (Gary and Clarke, 1998).

In budding yeast there appears to be only one protein arginine methyltransferase (Hmt1/Rmt1). Deletion of the *HMT1* gene eliminates all N<sup>G</sup>N<sup>G</sup>-dimethylarginine residues (Zobel-Thropp et al., 1998). Methylation of shuttling hnRNP proteins Npl3 and Hrp1 facilitate their export out of the nucleus. Both proteins bind poly A<sup>+</sup> RNA and their methylation-dependent nuclear export is correlated with a nuclear accumulation of poly

A<sup>+</sup> RNA, implicating methylation in the export of mRNA in yeast. Deletion of the HMT1 gene is not lethal; instead it results in a slow growth phenotype (Gary et al., 1996).

CBP80 and CBP20 form the cap binding complex (CBC) associated with the 5' trimethylguanosine cap of mRNA. The CBC mediates efficient pre-mRNA splicing, as well as snRNA export. Yeast strains bearing disruptions in *NPL3* and cap binding protein 80 (CBP80) are viable. However, deletion of the *HMT1* gene in these strains results in a lethal phenotype (Shen et al., 1998). The synthetic lethality of the *HMT1* deletion in  $\Delta$ *CBP80* and  $\Delta$ *NPL3* backgrounds, suggests that Cbp80p, Hrp1p, Npl3p all act in a linear pathway for the transport of RNA, each step being affected by Hmt1p. However, export of another methylated hnRNP shuttling protein, Hrb1, was not affected by deletion of HMT1 (Henry and Silver, 1996; Shen et al., 1998). So, the effects of methylation are still not clear.

HMW FGF is another protein that contains aDMAs. It has classic RGG motifs within an internal GAR domain. Methyltransferase inhibitors have implicated methylation in shifting the intracellular localization of HMW FGF from the nucleus to cytoplasm (Pintucci et al., 1996). The nuclear protein hnRNP A2 is involved in mRNA processing and splice site selection. It contains classic RGG motifs that are asymmetrically dimethylated *in vivo*. Removal of the GAR domain or inhibition of SAM dependent methylation shifts the cellular localization of hnRNP A2 from the nucleus to the cytoplasm, again suggesting a role for aDMA in subcellular localization (Nichols et al., 2000).

The results presented in Chapter 2 demonstrated that the GAR domain of hamster nucleolin is required for proper localization, indicating that the GAR domain is involved in targeting of wild type nucleolin to nucleoli. The results presented in Chapter 3 supported this idea by demonstrating a similar redistribution of nucleolin to the nucleoplasm in response to the methyltransferase inhibitors, MTA and DAA. Additionally, previous results by others demonstrated a potential link between methylation and the cellular localization of two of the aDMA-containing proteins, HMW FGF-2 and hnRNP A2 (Pintucci et al., 1996; Nichols et al., 2000). Experiments described in this chapter directly tests the hypothesis that methylation is required for nucleolin's localization to nucleoli.

## **MATERIALS AND METHODS**

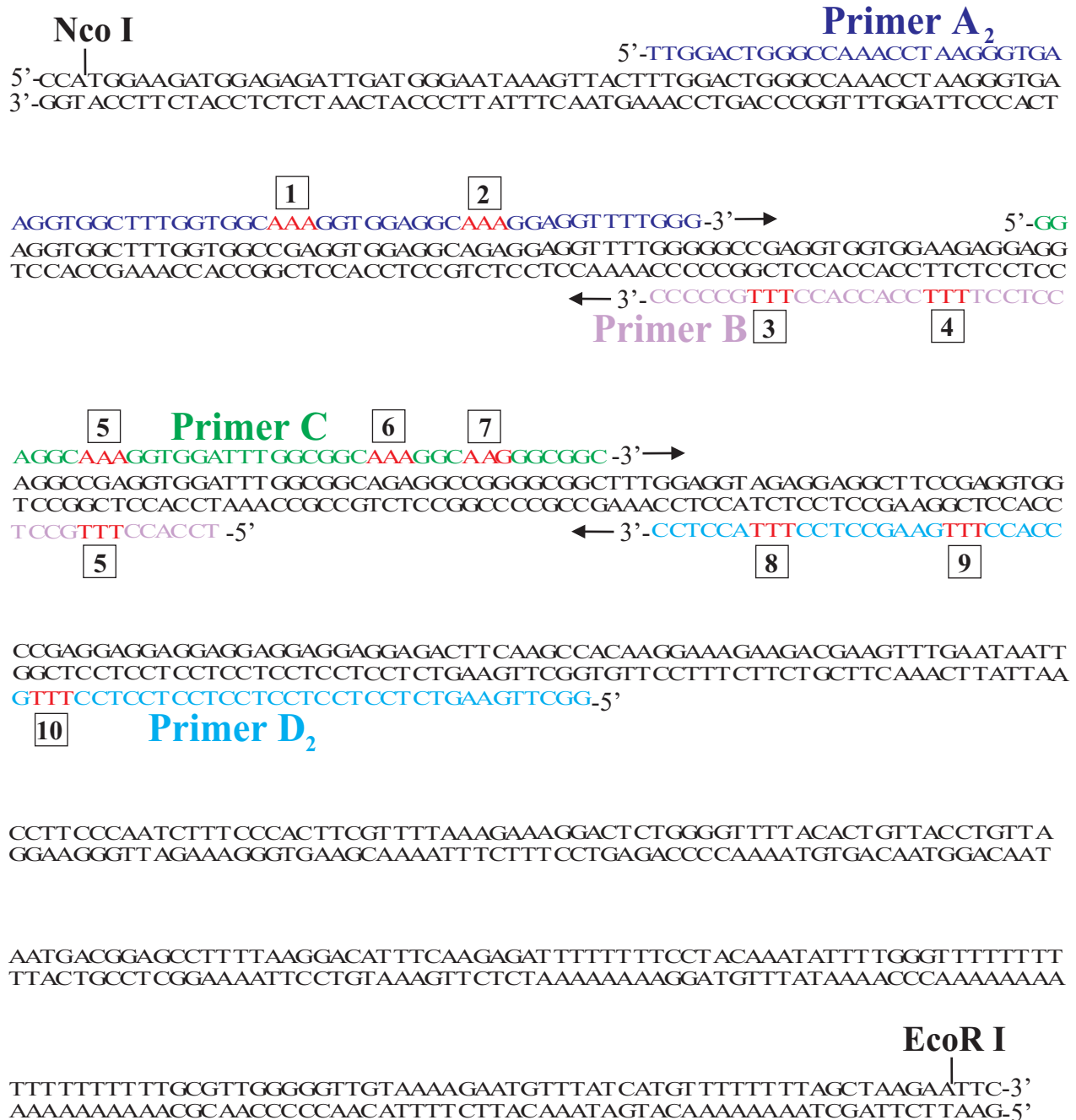
### **Generation of the Arg→Lys Substituted Mutant of Chinese Hamster Nucleolin**

The plasmid, 21R CHO WT (see Chapter 2 and Appendix B), was digested with Nco I and EcoR I at an internal Nco I site (+1886) and a C-terminal EcoR I site located in the polylinkers region of 21R CHO WT to generate a 452 bp DNA fragment encoding the GAR domain and the 3' UTR. The fragment was ligated into *myc-6-D* digested with the same restriction enzymes. Ligations and transformations were preformed following standard protocols. The final construct was called 21R CHO GAR WT (see Appendix B). All mutagenesis was preformed using this 21R CHO GAR WT subclone and its subsequent derivatives. The codons for all ten arginines within the GAR domain of the hamster nucleolin cDNA were changed to encode lysine. Oligonucleotides encoding the site-specific mutations were synthesized at GeneLab, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA. The four oligonucleotides named A<sub>2</sub> (5'-

GCCAAACCTAAGGGTGAAGGTGGCTTTGGTGGCAAAGGTGGAGGCAAAGGA  
GGTTTGGG -3'), B (5'-TCCACCTTTGCCTCCTCCTTTTCCACCACCTTGCCCC -  
3'), C<sub>2</sub> (5' - GGAGGCAAAGGTGGATTTGGCGGCAAAGGCAAGGGCGGC - 3'), and  
D<sub>2</sub> (5' - GGCTTGAAGTCTCCTCCTCCTCCTCCTTTGCCACCTTTGAAGCCTC  
CTTTACCTCC - 3') covered arginines 1-2, 2-3, 4-7 and 8-10, respectively (see Fig 4.1).  
A variation of the polymerase chain reaction (PCR) first described by Mullis et al. (1986)  
was used to make the base changes.

The first PCR reaction used 21R CHO WT GAR as template and primers A<sub>2</sub> and  
B to generate megaprimers. The megaprimers were then used in a second PCR reaction  
to generate a full length product that should have had sites 1-5 changed to encode lysine.  
After sequencing, it was realized that the product of the two step PCR reaction was  
heterogeneous, with one strand containing the A series (arginines 1 and 2) and the other  
strand containing the B series (arginines 3-5) (see Appendix B). The PCR product was  
subcloned back in *myc-6-D* using the Nco I and EcoR I sites, and the ligated product was  
introduced into competent *E. coli* using standard protocols. When the plasmid (21R  
CHO GAR B) was replicated in *E. coli*, the mismatched bases were recognized by the  
cell's DNA repair machinery and randomly repaired based on one of the two strands. In  
the case of sites 1, 2, and 5, the strand encoding the wild type sequence was used as the  
template. In the case of sites 3 and 4, the mutagenized strand was used as the template,  
thus changing the arginine codons to lysine.

A subsequent PCR reaction used 21R CHO GAR B as the template with primers D<sub>2</sub>  
(arginines 8-10) and T<sub>7</sub> in the polylinker of the plasmid. Primer D<sub>2</sub> contained a BsmA I  
site used for cloning purposes. The PCR product was subcloned (using Nco I and



**Figure 4.1** Location and orientation of primers used in PCR-based site directed mutagenesis. The sequence represents the entire subcloned GAR cDNA ligated into *myc-6-D*. The nucleotides in red (X) represent introduced mutations in the cDNA corresponding to Arg→Lys substitutions. Each arginine substitution is designated with a boxed number.

BsmA I) into *myc*-6-D and named 21R CHO GAR B/D. The next PCR reaction used 21R CHO GAR B/D as the template and primers A<sub>2</sub> (arginines 1 and 2) and T<sub>3</sub> in the polylinker of the plasmid. Primer A<sub>2</sub> contained a Bsu36 site used for cloning purposes. The resulting PCR product was cloned into *myc*-6-D and named 21R CHO GAR A/B/D.

The final PCR reaction used 21R CHO GAR A/B/D as the template. Primer C (arginines 5-7) contained no useful cloning site. Therefore, primer C and primer T<sub>3</sub> from the polylinker of the plasmid were used to create a megaprimer during the first 15 PCR cycles after which primer T<sub>7</sub> was added. Two PCR products were generated: those that had a wild type C series (arginines 5-7) due to the amplification during the first 15 cycles and those that had a mutagenized C series (see Appendix B)

PCR reactions were carried out in a Perkins-Elmer Cetus DNA Thermal Cycler. Final primer concentrations for all reactions were 1-2  $\mu$ M, and template DNAs were approximately 50 ng of a 812 base pair Pvu II - Pvu II fragment from the various 21R CHO GAR subclones. Vent<sup>®</sup> DNA polymerase was purchased from New England Biolabs (Beverly, MA). Buffer and dNTP concentrations were set according to New England Biolab's recommended protocols for Vent<sup>®</sup> DNA polymerase. The cycling program was: 94°C for 5 minutes, 55°C for 1 minute, and 72°C for 20 minutes for one cycle, followed by 40 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 3 minutes and 30 seconds. A final extension at 72°C for 15 minutes was used for all PCR reactions. Cloning and transformations were performed according to standard protocols. Single stranded DNA from mutagenized clones was prepared using M13 helper phage according to Ausubel et al. (1993). Site mutations were confirmed by dideoxy sequencing using a

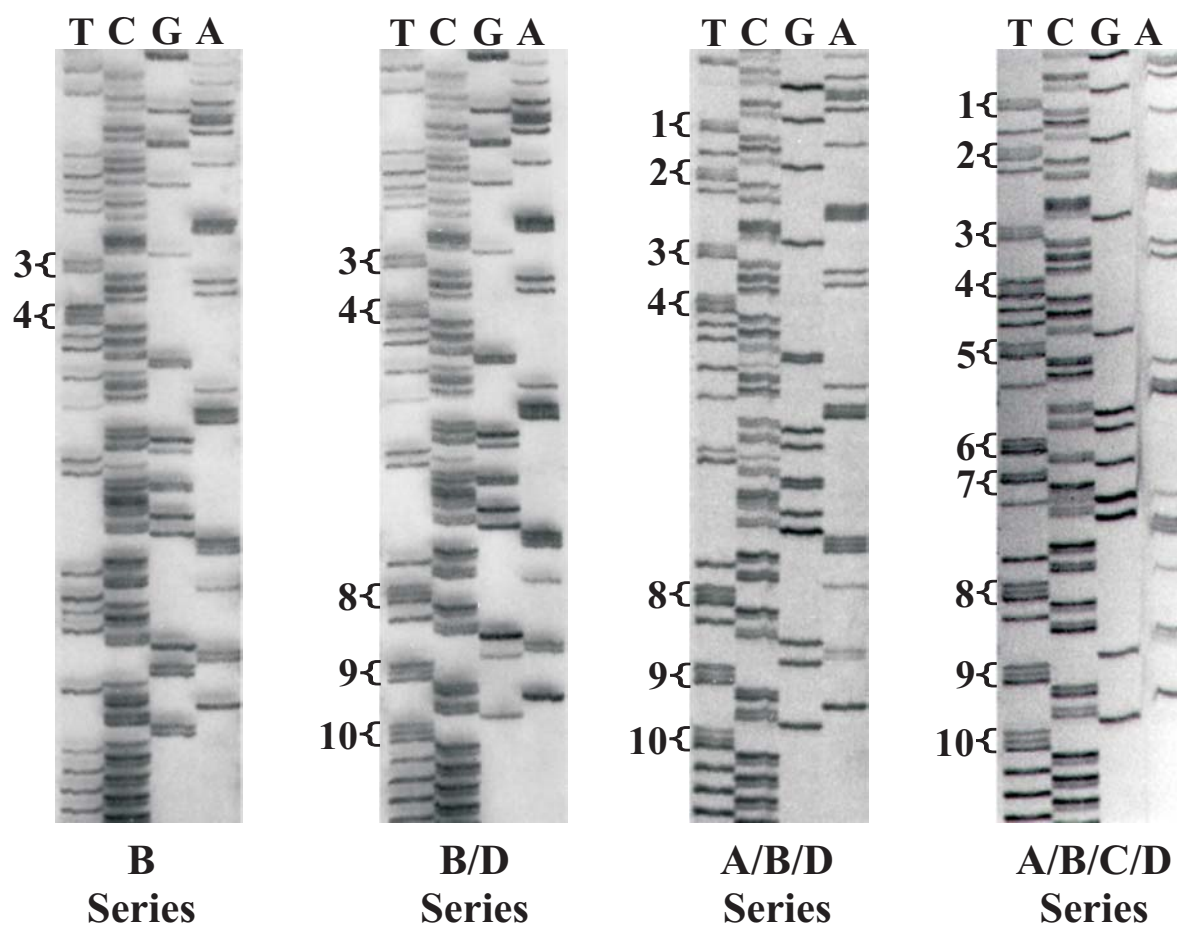
United States Biochemical (Cleveland, OH) Sequenase 2.0 kit, following the manufacture's recommended protocols (see Fig 4.2).

After sequencing to confirm the steps of mutagenesis, only the fully substituted GAR domain was pursued. Both the wild type (21R CHO GAR) and Arg→Lys substituted GAR subclones (21R CHO GAR A<sub>2</sub>BCD<sub>2</sub>) were digested with Nco I and EcoR I to liberate a 452 base pair fragment containing the respective GAR domains. The fragments were gel purified and ligated into the pET30a expression vector (Novagen, Madison, WI) that had been previously digested with the same restriction enzymes. Ligation placed the respective GAR domains behind and in frame with a histidine tag. The 452 base pair fragment from 21R CHO GAR A<sub>2</sub>BCD<sub>2</sub> was also cloned back into the full length nucleolin cDNA at the Nco I (+1886 in CHO cDNA) and EcoR I sites in 21R CHO. To allow for expression in eukaryotic systems, the DNA encoding the *myc*-tagged Arg→Lys substituted mutant nucleolin in 21R CHO MUT was cloned into the expression vector, pBK-CMV (Stratagene, La Jolla, CA) using Xho I and Xba I sites common to both polylinker regions.

### **Hmt1p and GAR Peptide Purification From *E. coli***

The yeast protein arginine methyltransferase, Hmt1p/Rmt1 (Henry and Silver, 1996), from *Schizosaccharomyces cerevisiae*, was a kind gift from Dr. Michael Henry. The HMT1 cDNA was provided in the pGEX 4T-1 vector (Pharmacia Peapack, N.J.) behind and in frame with a Glutathione-S-transferase (GST) tag. For compatibility with the affinity purification system used in our laboratory, the HMT1 cDNA was moved from the pGEX vector and ligated into the pET30a expression vector (Novagen, Madison WI). Specifically, HMT1 was removed from pGEX at the BamH I and Xho I restriction sites





**Figure 4.2** Autoradiograms of sequencing gels confirming the specific changes in the cDNA that encodes the GAR domain of Chinese hamster nucleolin. The numbers indicate the sites of the arginine to lysine codon conversion and correspond to the numbered sites in Figure 3.1 and Appendix B.

located in the polylinker regions of pGEX 4T-1. The resulting 1.9 kb fragment was ligated into pET30a which had been cut with BamH I and Xho I restriction endonucleases, placing the HMT1 cDNA behind and in frame with the DNA segment of pET30a that encodes a histidine tag. The resulting construct was named His-Hmt1.

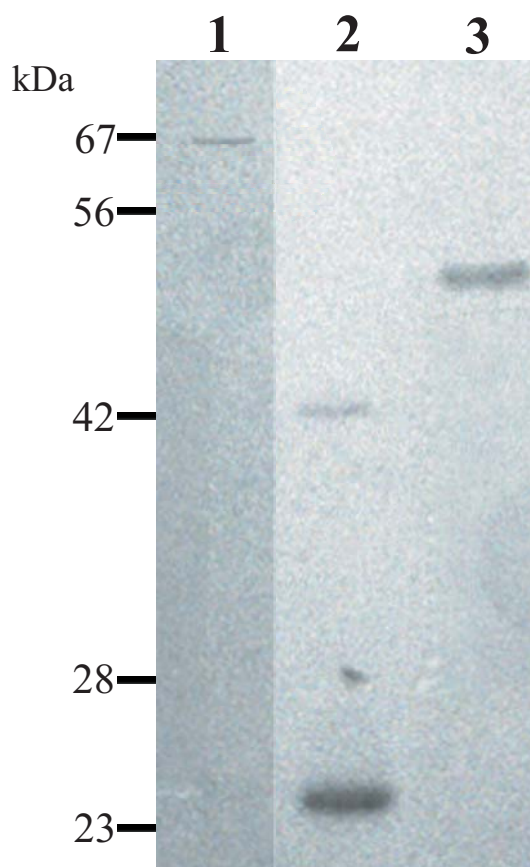
The His-Hmt1 construct was introduced into competent BL21(DE3)pLys S *E. coli* expression cells that were purchased from Novagen (Madison, WI). XL1-Blue and BL21(DE3)pLys S cells containing pGEX-Hmt1 and His-Hmt1, respectively, were grown to an O. D.<sub>600 nm</sub> of 0.65 in Lauria Bertani (LB) medium containing 100 µg/ml ampicillin for the pGEX vector or 30 µg/ml kanamycin and 15 µg/ml chloramphenicol for the pET30a vector. Cultures were then induced with 0.5 mM or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) respectively, for 3.5 hours at 37° C. Cells were pelleted at 6000 x g at 4° C for 15 minutes and then resuspended in 15 mls of 50 mM Tris [pH 8.0], 2 mM EDTA per 100 mls of original culture volume. The cells were repelleted, and stored overnight at -20°C as a semi-dry pellet. Upon thawing, the cells were resuspended in 1 ml (per 50 ml of original culture) of 1 x binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCL [pH 7.9]). Cells were lysed by sonication with six 30 second pulses on a setting of 4 at 50% duty cycle (Heat Systems-Ultrasonics Cell Disruptor, model CW-225 R). The lysate was centrifuged at 20,000 x g for 15 minutes to pellet cell debris, and the soluble fraction (supernatant) was passed through a 0.45 µm filter (Millipore, Bedford, MA). The filtrate was passed through the appropriate affinity column, either a glutathione sepharose 4B pre-packed column (Pharmacia Peapack, N.J.) or a His-bind resin column (Novagen, Madison, WI) (1 ml of bed volume per 50 mls of original culture). The columns were washed with 25 mls of binding buffer, and then 15

mls of wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris [pH 7.9]). The GST-Hmt1 fusion protein was eluted from the glutathione sepharose column with 5 mls of 10 mM glutathione, 50 mM Tris [pH 8.0]. The His-tagged Hmt1p was eluted with 15 mls of elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl [pH 7.9]). All buffers contained 0.5 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100. The eluant was dialyzed twice for three hours against two liters of 500 mM NaCl, 2 mM EDTA, 50 mM Tris [pH 8.0], after which the protein was concentrated 10-fold by placing the protein solution in a dialysis bag and placing the bag on polyvinylpyrrolidone (PVP, molecular weight = 360,000). The protein solution was next dialyzed against a reaction buffer (150 mM NaCl, 25 mM Tris [pH 8.0], 2.5 mM CaCl<sub>2</sub>, 0.1%  $\beta$ -mercaptoethanol, 10% glycerol). Aliquots of purified Hmt1p fusion proteins were resolved on a SDS-12% PAGE gel to determine purity (see Fig 4.3).

The same procedures were used to purify the His-tagged wild type and Arg→Lys substituted mutant GAR peptides (amino acids 628-733 of the full length protein), except that the final dialysis buffer was 500 mM NaCl, 50 mM Tris [pH 8.0]. Final protein concentrations were determined using a modified Lowry assay with BSA as the standard (Markwell et al., 1978).

#### ***In vitro* [<sup>3</sup>H]-SAM Methylation Assay**

Approximately 100 ng of purified recombinant His-Hmt1p was incubated with 1  $\mu$ g of wild type or 3  $\mu$ g of mutant His-tagged GAR peptides in the presence of 30  $\mu$ M S-adenosyl-L-[methyl-<sup>3</sup>H]methionine (SAM) with a specific activity of 14.7 Ci/mmol (Amersham, Piscataway, NJ). The methylation reaction was carried out at 30° C in 50 mM Na-MOPS [pH 7.2], 2 mM EDTA and 0.3 M NaCl for 40 minutes. Three fold



**Figure 4.3** Coomassie stained SDS-PAGE displays purified recombinate Hmt1p. Lane 1 is purified GST-Hmt1p. The GST tag was cleaved from GST-Hmt1p using thrombin (lane 2). The upper band in lane 2 is Hmt1p. The lower band in lane 2 is the cleaved GST tag and thrombin co-migrating together. Lane 3 contains the His-tagged version of Hmt1p. Protein molecular weight markers are indicated on the left.

concentrated SDS-gel sample buffer was added, and the samples were boiled for 5 minutes to stop the reaction. A mock reaction using the wild type peptide contained everything except His-Hmt1p. The samples were resolved on a SDS-10% PAGE. Following electrophoresis the gel was stained with Coomassie Blue and then fluorographed with Enhance (New England Nuclear, Boston MA), dried, and exposed to Kodak high speed X-ray film overnight.

***In Vitro* Translation of Wild Type, Arg→Lys Substituted, and ΔGAR Nucleolin.**

pBK-CMV (Stratagene, La Jolla, CA) containing *myc*-tagged wild type nucleolin, the *myc*-tagged Arg→Lys substituted mutant, or the *myc*-tagged ΔGAR nucleolin cDNAs was digested with Sma I to yield linear templates for *in vitro* transcription. The linear templates were gel purified by phenol:chloroform extraction followed by ethanol precipitation. Capped messenger RNA was *in vitro* transcribed using Ambion's T<sub>7</sub> mMessage Machine<sup>®</sup> kit following the manufacture's recommended protocols. The *in vitro* transcription reactions were treated with RNase-free DNase I supplied with the kit to remove the DNA template. Transcribed messenger RNA was phenol:chloroform extracted and ethanol precipitated. An aliquot of the capped mRNA was resolved on a discontinuous 1.5% agarose:formaldehyde gel to assess the quality and quantity of the RNA. Roughly equivalent amounts of the capped mRNAs were added to translation competent rabbit reticulocyte lysates (Promega Corporation, Madison, WI). Translation reactions were performed according to the manufacture's recommended protocols, except that 1 μl of 1 mM unlabeled methionine was substituted for the required [<sup>35</sup>S] labeled methionine. The translation reaction was mixed with an equal volume of 1 x low SDS-sample buffer (1.3% SDS) without β-mercaptoethanol or dithiothreitol (DTT). Samples

were heated at 94°C for 90 seconds before loading on a 1.5 mm, SDS-10% PAGE (Hoeffer Mighty Small<sup>®</sup> gel, rig model number SC250 size 8 X 10 cm, Amersham Biosciences Buckinghamshire, England). Electrophoresis was performed for 1.5 hours at 150 volts. The gel was transferred to nitrocellulose (Millipore Bedford, MA) using a standard tank buffer system. The proteins were transferred to the nitrocellulose for three hours at a constant 0.5 amps. Transfer of the proteins was confirmed by the presence of pre-stained protein markers (Gibco-BRL Life Technologies, Gaithersburg, MD). The nitrocellulose blot was immediately placed in pre-binding buffer (25 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM HEPES (pH-8.0), 1.0 mM EDTA (pH-8.0), 1 mM DTT, 5% Carnation non-fat dry milk) for 1.5 hours at room temp with shaking. A random primed DNA probe was prepared using Rsa I fragments of pBR322. The DNA probe had a specific activity of  $1.3 \times 10^5$  CPM/ $\mu$ l. The blot was then transferred to binding buffer (50 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1.0 mM DTT, 10 mM HEPES, 0.25% Carnation non-fat dry milk) and incubated with a  $4.5 \times 10^5$  counts of a [<sup>32</sup>P] labeled DNA probe for 1.5 hours. The blot was lightly dried for 45 minutes in a vacuum oven set at 60°C, before affixing it to 3MM chromatography paper (Whatman, Fairfield, NJ). The blot was then covered with plastic wrap and exposed to a phosphor screen for 24 hours. The phosphor screen was read using a Storm phosphorimager (General Dynamics, Buckinghamshire, England). The nitrocellulose blot was re-wetted with 1 x PBS and pre-blocked in 3% Carnation non-fat dry milk in 1x PBS for one hour. The blot was then incubated for one hour in mouse anti-*myc* mAb 9E10 hybridoma supernatant followed by five washes in 1 x PBS. The blot was incubated with a 1:3000 dilution of goat anti-mouse monoclonal antibody coupled to horseradish peroxidase in 10% horse serum for one hour followed by

three washes in 1 x PBS. The blot was developed using 4-chloro-1-napthaol as the chromogenic reagent.

### **Expression of the Arg→Lys Substituted Hamster Nucleolin in HeLa Cells**

HeLa cells were grown on coverslips in six well culture plates at 37°C in a 5% CO<sub>2</sub> atmosphere. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Life technologies Gibco BLR, Gaithersburg, MD) supplemented with 10% fetal calf serum and 50 µg/ml gentamycin. HeLa cells were transfected with pBK-CMV expression vectors carrying either cDNAs encoding *myc*-tagged wild type hamster nucleolin or the Arg→Lys mutation. Transient transfection was preformed as described in Chapter 2. Cells were hypotonically shocked for 15 minutes using 0.15 x PBS (Zatsepina et al. 1997), and then allowed to recover from the shock for up to 2 hours in normal growth conditions (DMEM with 10% FCS at 37°C in 5% CO<sub>2</sub>) after which all cells were fixed and prepared for microscopy as described previously.

## **RESULTS**

In Chapter 3 methyltransferase inhibitors (MTA and DAA) caused a change in the sub-cellular localization of exogenously expressed nucleolin. MTA and DAA, however, block all SAM dependent cellular methylation, including DNA methylation, RNA methylation, and protein methylation. The redistribution of nucleolin may, therefore, not be directly correlated to its methylation state. We set out to directly test the possibility that methylation of nucleolin's GAR domain is necessary for nucleolar association.

### **Generation and *In Vitro* Methylation of a Lysine Substituted GAR Domain**

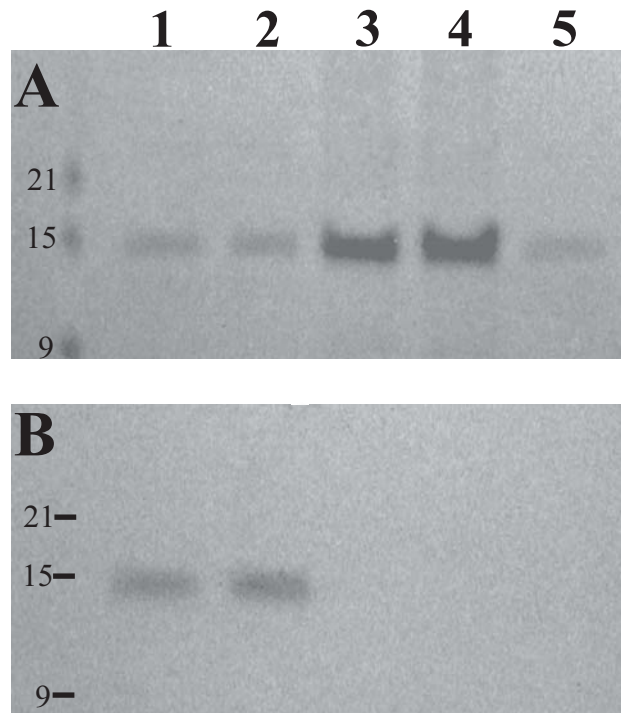
A Chinese hamster nucleolin cDNA subclone, consisting of amino acids 650-733 was ligated into pBluescript and used for PCR-based oligonucleotide site directed

mutagenesis. All ten arginines within the GAR domain were changed to lysine. In addition to generating the fully substituted nucleolin, the technique also yielded a series of partially substituted nucleolins. This series was originally designed to test the effect of degrees of methylation if the fully substituted protein displayed a distinct phenotype. The changes were confirmed by dideoxy sequencing (see Fig. 4.2). The conservative change to lysine maintains the relative charge and size of the side chain.

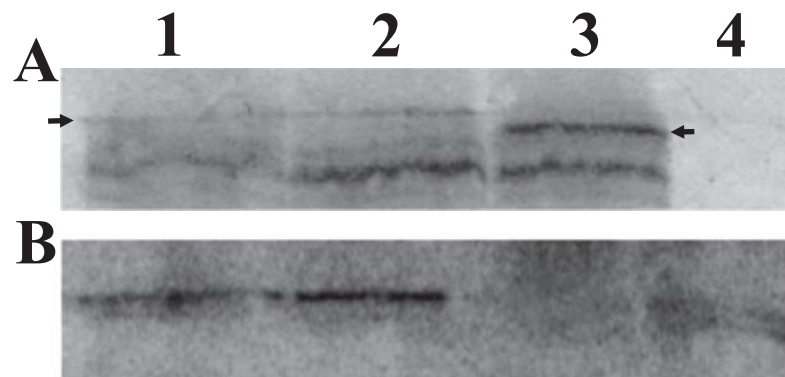
Like the terminal guanido nitrogens of arginine, the  $\zeta$ -nitrogen of lysine can be post-translationally methylated. However, lysines were observed not to be methylated under conditions permissive for arginine methylation (see below). To confirm that the fully substituted Arg→Lys GAR domain of nucleolin was not a substrate for post-translational enzymatic methylation, a recombinant His-tagged version of the protein arginine methyltransferase from *Schizosaccharomyces cerevisiae*, Hmt1p, was purified for use in *in vitro* methyltransferase assays (see Fig. 4.3).

Wild type and the fully Arg→Lys substituted carboxy terminal domains of nucleolin (amino acids 628-733) were expressed in *E. coli* as amino terminal His-tagged fusion proteins. The peptides were affinity purified using  $\text{Ni}^{2+}$  columns. Peptides were incubated with Hmt1p in the presence of S-adenosyl-L-[methyl- $^3\text{H}$ ]-methionine as the methyl donor. The wild type peptide is readily methylated by Hmt1p (see Fig. 4.4, panel B, lanes 1 and 2). The mutant peptide, however, shows no detectable signal (see Fig. 4.4, panel B, lanes 3 and 4) even though it is present in four-five times the quantity of the wild type peptide (see Fig. 4.4, panel A, lane 3 and 4). A mock reaction containing the wild type peptide incubated in the presence of [methyl- $^3\text{H}$ ]-SAM without Hmt1p, showed no detectable signal (see Fig. 4.4, lane 5). Therefore, the GAR peptide contains no





**Figure 4.4** Hmt1p methylation of wild-type and the fully lysine substituted GAR peptides. Panel A is a Coomassie stained PAGE-gel. Panel B is the autoradiogram of panel A. Wild type and lysine substituted peptides were expressed in *E. coli* as His-tagged peptides. The wild type and mutant peptides were incubated with recombinant Hmt1p and S-adenosyl-L-[methyl- $^3\text{H}$ ]methionine ([methyl- $^3\text{H}$ ]SAM) (wild type lanes 1 and 2, mutant lanes 3 and 4). The wild-type peptide was also incubated with [methyl- $^3\text{H}$ ] SAM alone (lane 5). Approximately 4-fold more lysine substituted mutant peptide (Panel A lanes 2 and 3), was present compared to the wild type peptide (Panel A lanes 1 and 2). Only the wild-type peptide was methylated (Panel B lane 1 and 2). The wild-type peptides ability to be labeled was dependant on the presence of Hmt1p since no signal was detected when the wild-type peptide was incubated with [methyl- $^3\text{H}$ ] SAM alone (Panel B lane 5).



**Figure 4.5** Southwestern binding assay of *myc*-tagged wild type nucleolin, the lysine substituted protein and  $\Delta$ GAR nucleolin. Panel A is an immuno-blot against the *myc*-tags. The right facing arrow indicates full length nucleolins (Panel A, lane 1). The left facing arrow indicates  $\Delta$ GAR nucleolin. Panel B is the autoradiogram of the blot in Panel A. Wild type nucleolin and the mutant protein bound radiolabeled DNA (Panel B, lanes 1 and 2, respectively). Although  $\Delta$ GAR nucleolin was approximately 10-20 fold (panel A, lane 3) in excess over wild type protein, it exhibited no nucleic acid binding above background (panel B, lane 3). Lane four is a control to account for lysate proteins. An endogenous nucleic acid binding protein co-migrated with  $\Delta$ GAR, and likely accounting for the smeared signal (panel B, lane 3).

endogenous methylation activity, and methylation of the wild type peptide is dependant only on the presence of Hmt1p. Taken together, these results show that the lysine substituted mutant is not a substrate for RGG specific methyltransferase, Hmt1p.

### **Nucleic Acid Binding Ability of Wild Type, Arg→Lys Substituted, and ΔGAR Nucleolin**

Messenger RNA encoding *myc*-tagged wild type, lysine substituted, and ΔGAR nucleolins were expressed separately in rabbit reticulocyte lysates. The expressed proteins were transferred to nitrocellulose. The blot was incubated with non-specific [<sup>32</sup>P]-labeled double-stranded DNA. Slightly more mutant protein was produced than wild type (see Fig. 4.5, lanes 1 and 2). The GAR truncated nucleolin was in excess of the wild type protein by approximately 5-10 fold (Fig. 4.5, lane 3). Lane 4 in Figure 4.5 is a control to account for endogenous translation products; no mRNA was added.

Quantification of the radioactive signals associated with the wild type and mutant proteins shows that the mutant signal was approximately 10% stronger than the wild type signal (Fig. 4.5, panel B, lanes 1 and 2). The ΔGAR nucleolin co-migrated with an endogenous nucleic acid binding protein. Therefore, the nucleic acid binding of ΔGAR could not be quantified. It appears from the autoradiogram, however, that the ΔGAR signal was equivalent of background (Fig 4.5, panel B, lanes 3 and 4).

### **Expression of the Fully Substituted Arg→Lys Nucleolin in Interphase HeLa Cells**

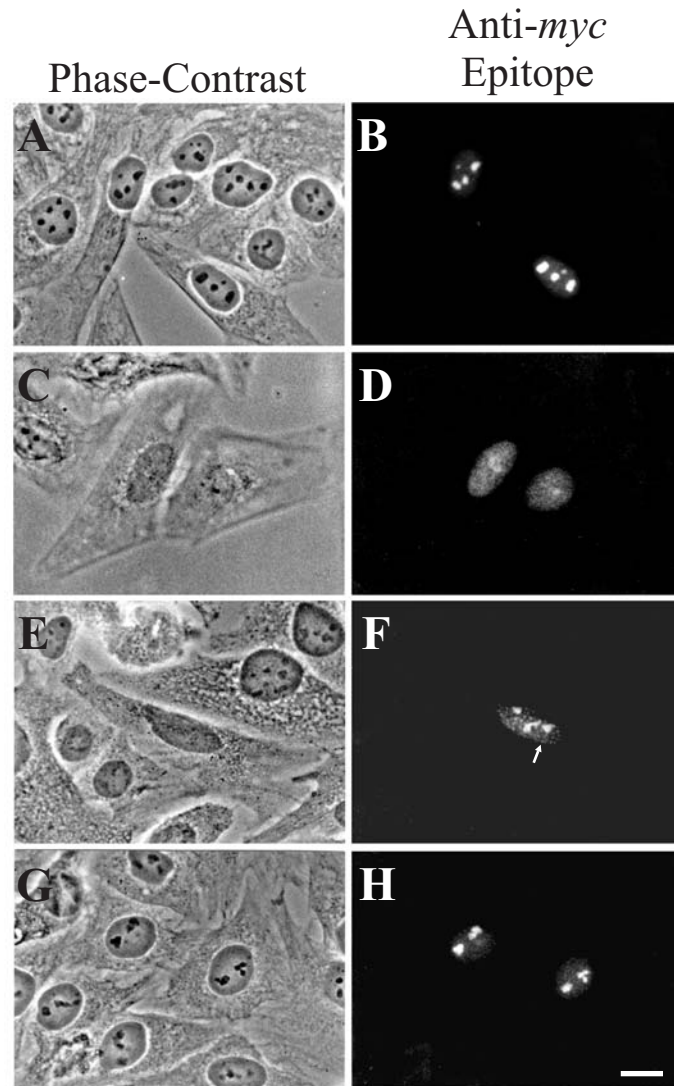
Chapter 2 demonstrated that the GAR domain of nucleolin was necessary for nucleolar localization. Arginines located within the GAR domain have been shown to be post-translationally dimethylated (Lischwe et al., 1981; Lischwe et al., 1982). When cellular methylation was blocked using methyltransferase inhibitors, nucleolin underwent a rapid redistribution from nucleoli to the nucleoplasm in interphase cells. This result is

consistent with results showing the mis-localization of HMW FGF2 and hnRNP A2 when their methylation was blocked by the same inhibitors. Blocking methylation redistributed HMW FGF2 and hnRNP A2 from the nucleus to the cytoplasm (Pintucci et al. 1996; Nichols et al., 2000). In addition, reviews on nucleolin suggest that methylation may play a role in its localization to nucleoli (Ginisty et al. 1999). A version of nucleolin which could not be methylated was used to directly test the question: is methylation necessary for proper localization?

Full-length, *myc*-tagged, wild type and Arg→Lys substituted nucleolin was expressed in HeLa cells by transient transfections. The fully substituted nucleolin properly localized to interphase nucleoli as determined by antibody staining (see Fig. 4.6, A and B). The staining pattern and nucleolar morphology was indistinguishable between cells expressing the wild type protein and the fully substituted protein.

Exposing cells to hypotonic conditions causes disassembly of interphase nucleoli. Hypotonically shocked cells redistribute their nucleolar components to the nucleoplasm (Zatsepina et al., 1997). Zatsepina et al. (1997) demonstrated that return of cells to normal osmotic growth conditions after hypotonic shock causes nucleoli to reform. The process of reforming nucleoli is accompanied by the appearance of discrete extranucleolar bodies, that are very similar to pre-nucleolar bodies (PNBs) observed in teleophase of mitosis.

HeLa cells expressing wild type and Arg→Lys substituted nucleolin were hypotonically shocked to cause disassembly of interphase nucleoli. The behavior of the transfected nucleolins was monitored as nucleoli reformed (Fig. 4.6, C-F). Both proteins associate with interphase pre-nucleolar bodies (iPNBs) first, and then with fully reformed



**Figure 4.6** HeLa cells were transfected with cDNAs encoding *myc*-tagged fully lysine substituted Chinese hamster nucleolin. The *myc*-tagged lysine substituted mutant nucleolin localized to interphase HeLa cell nucleoli (**A** and **B**). The transfected cells were subjected to 15 minutes of hypotonic shock in 0.15 x PBS, causing a breakdown of the interphase nucleoli and redistribution of the lysine substituted mutant nucleolin (**C** and **D**). After one hour of recovery in fresh medium, the lysine substituted mutant nucleolin is observed in pre-nucleolar bodies (arrow in **F**) and reforming nucleoli (**F**). Nucleoli had fully reformed after two hours of recovery and the lysine substituted mutant nucleolin had reassociated with the nucleoli (**G** and **H**). Calibration bar is 20  $\mu$ m for all panels.

nucleoli (see Fig 4.6, G-J). The behavior and kinetics of reassembly of nucleoli between the wild type nucleolin and Arg→Lys substituted protein were indistinguishable, thus demonstrating that early associations of nucleolin with other nucleolar proteins and partial processed pre-rRNA in the absence of rDNA are unaffected by the substitution of lysine for arginine in the GAR domain of hamster nucleolin.

## DISCUSSION

The results of Chapter 3 suggest that methylation may play an important role in the sub-cellular localization of nucleolin. This possibility is based on results obtained using methyltransferase inhibitors to inhibit SAM-dependent cellular methylation. However, such inhibitors are not only blocking arginine methylation, but also DNA methylation, RNA methylation and other forms of protein methylation. The effects seen with these methyltransferase inhibitors may not be directly related to blocking methylation of the target protein, but instead the consequence of interfering with other cellular targets. It is important to determine if the *in vivo* effects observed using methyltransferase inhibitors are directly related to arginine methylation of the GAR domain of nucleolin. In order to determine this, a version of nucleolin that would not be methylated was generated.

All ten arginine residues present within the GAR domain of Chinese hamster nucleolin were changed to lysine. Of the twenty basic amino acids, the side chain of lysine is the closest in size and charge to that of arginine. The intrinsic pKa of the amino group in the lysine side chain is 11.1 compared to a pKa value of about 12 for the guanido nitrogens of arginine. Lysine is more chemically reactive under physiological

conditions due to having a higher percentage of nonionized side chains, which are potent nucleophiles compared to the arginine guanido group.

The  $\zeta$ -nitrogen of lysine can be post-translationally mono- or dimethylated *in vivo*. An *in vitro* methylation assay was used to determine if lysine in the context of a RGG domain would be recognized as a substrate for RGG-specific protein arginine methyltransferases. The yeast, *Schizosaccharomyces cerevisiae*, appears to have only one protein arginine methyltransferase, Hmt1p. All the known RGG containing proteins in yeast are substrates for this enzyme, indicating that it is able to recognize a broad range of RGG-containing substrates (Henry and Silver, 1996; Lin et al., 1996). In vertebrates, there are six different genes that encode protein arginine methyltransferases, five of which catalyze the formation of aDMA. The existence of multiple protein arginine methyltransferases in vertebrates indicates a much higher level of specificity than exists in yeast, which contains the one protein arginine methyltransferase (Frankel and Clarke, 2000; Tang et al., 2000; Frank et al., 2001; Rho et al., 2001). If Hmt1p is unable to methylate lysine in the KGG motifs of the lysine substituted nucleolin GAR domain, then it is highly unlikely that any of the more specific protein arginine methyltransferases of vertebrates would be able to methylate the lysine residues in the KGG motifs.

A peptide of the wild type GAR domain proved to be an excellent substrate for the protein arginine methyltransferase, Hmt1p, as determined by radiolabel incorporation of the methyl group from S-adenosyl-L-[methyl-<sup>3</sup>H]-methionine. The lysine substituted peptide, however, was not a suitable substrate for Hmt1p (see Fig. 4.4). The wild type peptide showed no detectable signal without the presence of Hmt1p, indicating that methylation is dependant on the presence of Hmt1p (see Fig 4.4). These results

demonstrate that lysine in the context of a KGG motif is not a substrate for Hmt1p, and therefore unlikely to be a substrate for any of the more substrate-specific vertebrate protein arginine methyltransferases.

Removal of nucleolin's GAR domain greatly reduces nucleolin's nucleic acid binding ability. The nucleolar localization of nucleolin is thought to be due to functional association with nucleolar components, probably via its interaction with pre-rRNA. Therefore, it was important to determine the effects of the lysine substitutions on nucleic acid binding. A Southwestern binding assay was performed using wild type,  $\Delta$ GAR, and the fully substituted Arg $\rightarrow$ Lys nucleolins. All three proteins were expressed in rabbit reticulolysates. Nucleolin lacking its GAR domain failed to bind nucleic acids (see Fig 4.5, lane 3) in this assay supporting previous results (Heine et al., 1993). The lysine substituted nucleolin was able to bind nucleic acids in a Southwestern binding assay (see Fig 4.5, lane 2). This is somewhat surprising, because Raman et al. (2001) showed that *in vitro* synthesized KGG peptides exhibited 20-50 fold lower nucleic acid binding as compared to RGG peptides. Their study, however, used a small peptide corresponding to a portion the GAR domain from human nucleolin, and it may not reflect the behavior of GAR domains in the context of the full length protein with the four upstream RBDs.

The GAR domain is thought to modulate nucleolin's interaction with nucleic acids by disrupting secondary structure of the RNA, thus allowing access for the RBDs (Ghisolfi et al., 1992a; Hanakah et al., 2000). The exact mechanism of the GAR domain's ability to perturb RNA superstructure is not known. This function may not be disrupted by the substituted lysines, allowing for normal nucleic acid binding. This possibility is supported by the fact that the KGG peptides behaved identically to aDMA (RGG)



containing peptides with respect to their ability to perturb nucleic acid superstructure, which is remarkably different from the unmethylated peptide (Raman et al., 2001). The guanido group of arginine is planar as a result of resonance. Dimethylation of one of the nitrogens would result in a decrease in this resonance, and therefore, a loss of the planar configuration. This would cause the unmethylated nitrogen to resemble the amino group of lysine. In addition, dimethylation would reduce the number of hydrogens available for hydrogen bonding, and the available geometries for hydrogen bonding to the same as that of lysine (Raman et al., 2001). For these reasons, the lysine substituted nucleolin may behave in a similar manner to aDMA-containing nucleolin.

KGG and aDMA containing RGG peptides are known to modify nucleic acid conformation in a similar manner. If the sub-cellular localization is a function of the GAR domain's ability to perturb nucleic acid structure, lysines may preserve this function by mimicking the behavior of the aDMAs.

The lysine substituted nucleolin localized properly to interphase HeLa cell nucleoli (see Fig 4.6, A and B). The lysine substituted nucleolin was redistributed to the nucleoplasm by hypotonic shock, just as did the wild type protein. The behavior and kinetics of nucleolar reassembly was indistinguishable between cells expressing wild type nucleolin and the fully substituted protein. This result demonstrates that early associations of the Arg→Lys substituted protein with nucleolar RNAs or other proteins in the absence of rDNA are not affected (see Fig. 4.6, C-H).

The localization of the lysine substituted nucleolin disagrees with the results of the methyltransferase inhibitors presented in Chapter 3. The lysine substituted nucleolin is not a substrate for a RGG specific protein arginine methyltransferase, indicating that it

cannot be methylated *in vivo* (see above). The lysine substituted nucleolin was able to bind nucleic acids as determined by a Southwestern assay (see Fig. 4.5). The proper localization of the unmethylated, lysine-substituted nucleolin demonstrates that arginine methylation is not involved in the sub-cellular localization of nucleolin. The fact that the unmethylated lysine substituted nucleolin behaves normally *in vivo*, indicates that the redistribution of nucleolin in the presence of methyltransferase inhibitors, presented in Chapter 3, is an indirect consequence associated with the methyltransferase inhibitors used.

## Chapter 5

### Summary, Conclusions and Future Directions

Nucleolin was first described almost 30 years ago by Orrick et al. (1973) and its cDNA was cloned 17 years ago by Lapeyre et al. (1985). Since its first discovery, nucleolin has been implicated in a wide array of functions including rDNA transcription, pre-rRNA processing, ribosome assembly, nucleocytoplasmic transport, immunoglobulin gene switching, telomeric DNA association, insulin response, glucocorticoid receptor function, and transcription regulation (Csermely et al. 1993; Hanakahi et al., 1997; Nisole et al. 1999; Pollice et al., 2000; Schulz et al., 2001; Tuteja and Tuteja, 1998; Ginisty et al., 1999; Erard et al., 1988; Borer et al., 1989; Herrera and Olson, 1986; Bouvet et al., 1998). Nucleolin's primary function, however, is related to ribosome biogenesis (see Chapter 1). Nucleolin's role in ribosome biogenesis puts it at the heart of one of the cells most important activities. The nucleolus is proving to be "plurifunctional" and more than just a ribosome factory, with roles in post-translational modification and ribonucleoparticle assembly of non-ribosomal RNAs, gene silencing, aging and cell cycle control (Pederson, 1998b; Dundr and Misteli, 2001). These may be reasons why nucleolin is apparently involved in so many diverse cellular processes.

This dissertation demonstrates the requirement of the glycine-arginine-rich (GAR) domain for proper *in vivo* localization of hamster nucleolin. Nucleolin lacking the carboxy terminal GAR domain fails to localize to interphase nucleoli. Instead, it accumulates within the nucleoplasm (see Fig. 2.2). The nucleoplasmic localization of  $\Delta$ GAR was then used as a novel probe to test for interactions with other nucleolar proteins. Nucleolar proteins Nopp140 and fibrillarin showed little or no interaction with

$\Delta$ GAR. B23, however, redistributed to the nucleoplasm in the presence of  $\Delta$ GAR (see Figs. 2.3-2.5). This suggests that B23 associates with  $\Delta$ GAR nucleolin *in vivo*. This result is consistent with the interaction observed between nucleolin and B23 as determined by a yeast two hybrid assay and immuno-precipitation (Li et al., 1996).

The nucleolar accumulation of nucleolin is apparently the result of a complex interplay between several domains. RNase treatment induces the release of nucleolin from nucleoli in A6 cells confirming that nucleolin's localization at least requires the presence of RNA (Schwab and Dreyer, 1997). The RNA-binding domains (at least one) are required for nucleolar localization, but they are unable by themselves to target hybrid proteins to the nucleolus (Creancier et al., 1993). In conjunction with the RBDs, the GAR domain of nucleolin is also required for nucleolar localization, but it too cannot separately target a hybrid protein to the nucleolus (Heine et al., 1993; Schmidt-Zachmann and Nigg 1993; Creancier et al., 1993). In the case of the yeast protein, Nsr1, the N-terminal acidic / basic domain was able to direct a  $\beta$ -galactosidase fusion to yeast nucleoli (Yan and Mélése, 1993). This however, was not the case for the homologous region of vertebrate nucleolin (Schmidt-Zachmann and Nigg 1993; Creancier et al., 1993). So differences exist between the vertebrate and yeast "homologs".

The GAR domain of nucleolin consists several RGG the tripeptide motifs. It is repeated 10 times with interspersed aromatic amino acids (see Fig. 1.4). RGG motifs containing aDMAs are found in a number of RNA binding proteins (see Chapter 1). At least eight, and possibly all ten arginines in the RGG motifs are post-translational dimethylated *in vivo* (Lischwe et al., 1985; Lapeyre et al., 1986). The functional significance of arginine dimethylation in nucleolin is unknown.

Asymmetric dimethylation eliminates the planarity of the guanido group of the arginine side chain. The remaining unmethylated nitrogen would then resemble the amino group of lysine with the same number of hydrogen atoms available for hydrogen bonding. Evolution, however, has maintained arginines and not lysines in GAR domain-containing proteins from yeast to man. The high metabolic cost of arginine methylation (12 ATP per methylation event) would be eliminated by natural selection, unless it provided a needed function (Gary and Clarke 1998). Methylation does not change the overall charge of the RGG domain, but the addition of the bulkier methyl groups may modulate protein-protein interaction or protein-nucleic acid interactions (Tao and Frankel, 1992; Liu and Dreyfuss, 1995; Raman et al., 2001; Friesen et al., 2001). Since the GAR domain of nucleolin is known to be involved in the nucleolar localization and nucleic acid binding of nucleolin, we decided to explore what, if any role asymmetric arginine dimethylation would play in these activities.

The methyltransferase inhibitors 5-deoxy 5' methythioadenosine (MTA) and 3-deaza-adenosine (DAA) redistributed exogenously expressed nucleolin from nucleoli to the nucleoplasm in transfected HeLa cells (see Fig. 3.2). Nucleolin redistribution due to MTA was reversed upon removal of the methyltransferase inhibitor. The reassociation of nucleolin with nucleoli upon MTA removal was equally as rapid, taking as little as 1 hour (see Fig 3.3). Protein synthesis was blocked to determine if the affects observed with MTA could be the result of newly synthesized, unmethylated nucleolin entering the nucleus, but simply unable to associate with nucleoli. *Myc*-tagged nucleolin accumulated in the nucleoplasm in an identical manner in the presence of MTA when protein synthesis was blocked using cycloheximide (see Fig. 3.3). This suggests that nucleolin already

localized to nucleoli prior to MTA exposure redistributed to the nucleoplasm, and that the nucleolar localization of nucleolin may be dependent on arginine methylation.

The possibility that nucleolar localization of nucleolin may be dependent on arginine methylation is supported by the role of arginine dimethylation in HMW-fibroblast growth factor and hnRNP A2. Both proteins have been shown to change their sub-cellular localization from nuclear to cytoplasmic in the presence of methyltransferase inhibitors, MTA and DAA (Pintucci et al., 1996; Nichols et al., 2000).

Another nucleolar protein, fibrillarin, did not redistribute in the presence of MTA (see Fig 3.2). This is somewhat surprising since fibrillarin contains a GAR domain that is highly methylated *in vivo* (Christensen and Fuxa, 1988). The differences in the behavior of nucleolin and fibrillarin in the presence of MTA may reflect distinct functional differences between their respective GAR domains (see Chapter 3). The redistribution of nucleolin in the presence of the methyltransferase inhibitors, therefore, may not be correlated to its methylation, but rather to inhibiting methylation of another substrate, possibly pre-rRNA (see Chapter 3).

To directly test the possibility that methylation of nucleolin's GAR domain is necessary for nucleolar association, all ten arginines within the GAR domain of Chinese hamster nucleolin were substituted with lysine (see Fig 4.1). The protein arginine methyltransferase from *Schizosaccharomyces cerevisiae*, Hmt1p, was purified for use in an *in vitro* methyltransferase assay. Hmt1p is the sole Type I protein arginine methyltransferase in *Schizosaccharomyces cerevisiae*, and it is therefore able to recognize and methylate a broad range of substrates (see Chapter 4). Hmt1p was able to efficiently methylate the wild type GAR peptide (see Fig. 4.4, lanes 1 and 2). The fully

substituted GAR peptide, however, was not a substrate for Hmt1p (see Fig 4.4 lanes, 3 and 4). The results indicate that the amino nitrogen of lysine is not a substrate for protein arginine methylation *in vivo*.

The nucleolar localization of nucleolin is thought to be due to functional association with nucleolar components, namely pre-rRNA. To determine the affect of the Arg→Lys substitution on nucleic acid binding, a Southwestern assay was preformed. Both the lysine substituted protein and the wild type nucleolin were demonstrated to bind nucleic acids to roughly equivalent extents (see Fig 4.5).  $\Delta$ GAR nucleolin, however, failed to bind nucleic acids in this assay (see Fig 4.5) supporting previous results (Heine et al., 1993).

The Arg→Lys substituted protein was able to properly localize to interphase nucleoli when transiently expressed in HeLa cells (see Fig. 4.6). When nucleoli were disrupted by hypotonic shock, the Arg→Lys substituted protein reassembled with behavior and kinetics indistinguishable from that of cells expressing wild type nucleolin (see Fig. 4.6). The proper localization of the unmethylated, lysine-substituted nucleolin argues against the process of methylation being involved in the sub-cellular localization of nucleolin.

To understand the methyltransferase inhibitor results, it will be crucial to determine the effects of the methyltransferase inhibitors on cellular activities including pre-rRNA synthesis. The methyltransferase inhibitors resemble adenosine, and may therefore, be very toxic to the cell by inhibiting ATP-dependent events. Determination of the specificity and enzymology of these compounds (MTA and DAA) needs to be

addressed before data resulting from their use can be interpreted correctly and with confidence.

Arginine methylation is often associated with RNA-binding and no clear examples of the effect of arginine methylation in the context of fully methylated full-length protein have been examined. For this reason it would be of great benefit to precisely determining the binding of methylated and unmethylated wild type nucleolin as well as the lysine substituted protein to nucleolin's high affinity *in vivo* RNA binding sites (NRE). In addition to the methylation of the protein it is also possible that pre-rRNA must be methylated on its sugar to allow nucleolin to correctly interact with pre-rRNA *in vivo*.

Unfortunately, production of the unmethylated form of nucleolin requires production in prokaryotes which lack protein arginine methyltransferase activity. Despite great effort by myself and others, no one has yet successfully produced recombinant hamster nucleolin in great abundance due to its extreme cytotoxicity and instability. Perhaps the use of cell-free bacterial lysates systems or expression in the newly created type I methyltransferase minus background in yeast may be able to produce usable amounts of protein (Zobel-Thropp et al., 1998). The instability of recombinant nucleolin may be related to the lack of post-translational modifications, namely phosphorylation and methylation. Examining the effects of these post-translational modifications on nucleolin stability or targeted degradation may reveal one of their possible functions.

One obvious question not yet answered relating to nucleolin's arginine methylation is where and when does nucleolin methylation take place? The most likely candidate for arginine methylation in vertebrates is PRMT1, which is primarily located in



the nucleus. If the post-translational methylation of nucleolin is irreversible, then why should the protein arginine methyltransferase be nuclear, unless the temporal control of methylation is important and the nascent protein is not immediately methylated after translation in the cytoplasm. Pulse chase experiments could be used to determine the timing of methylation of nucleolin *in vivo*.

As interest in protein arginine methylation grows, so does the understanding of its importance to cellular function. The number of known substrates for protein arginine methylation is ever increasing. Models for the function of arginine methylation in nucleolin will improve as new and novel roles of arginine methylation in other proteins are elucidated.

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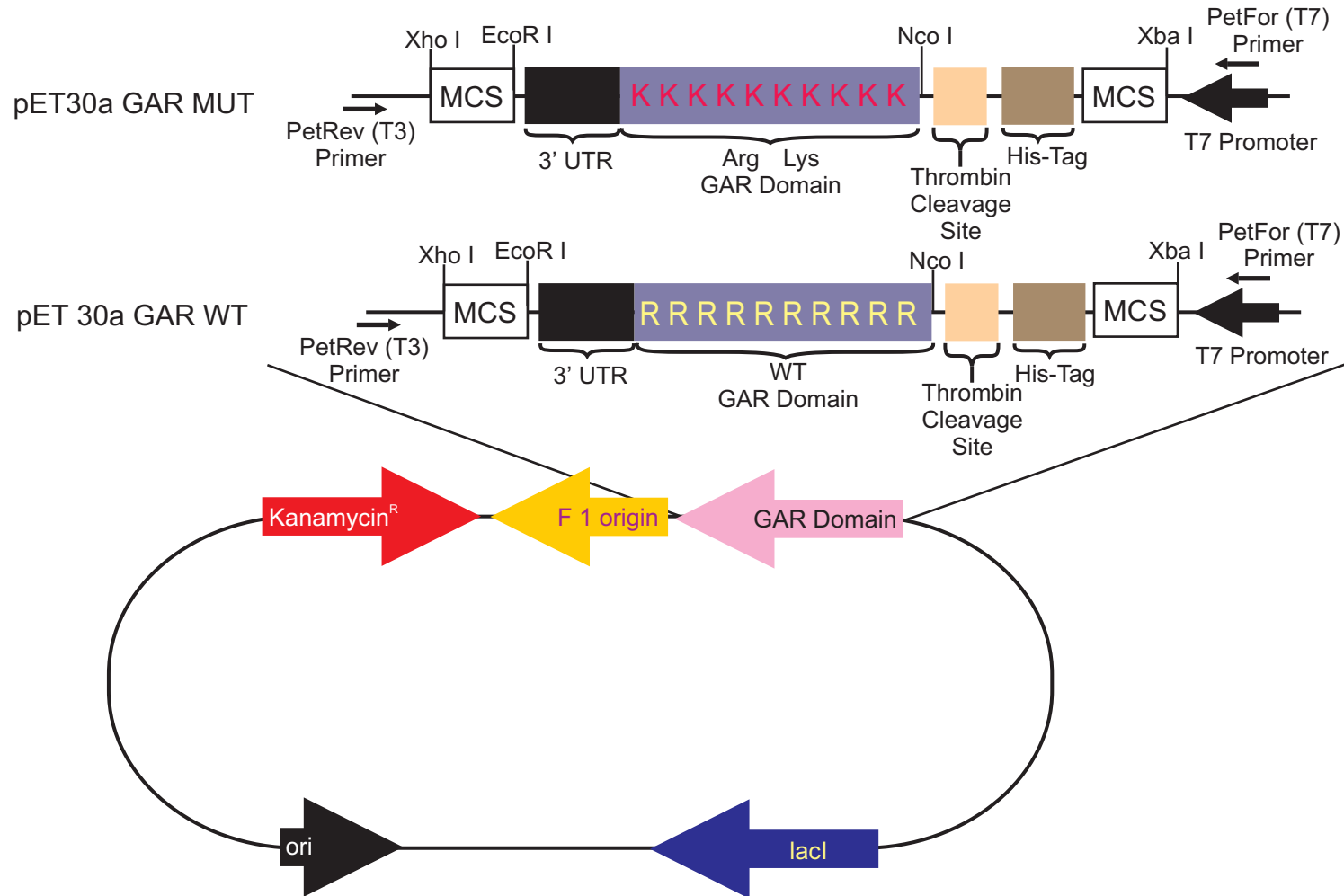
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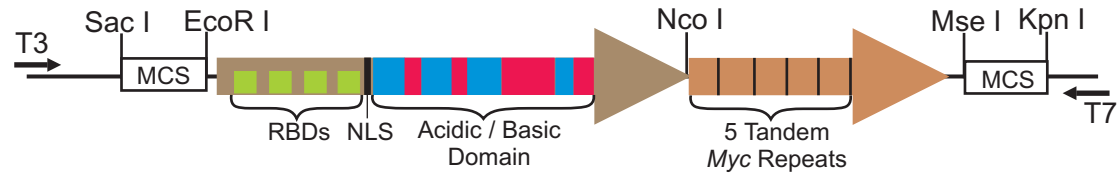
## Appendix A: Vector Maps



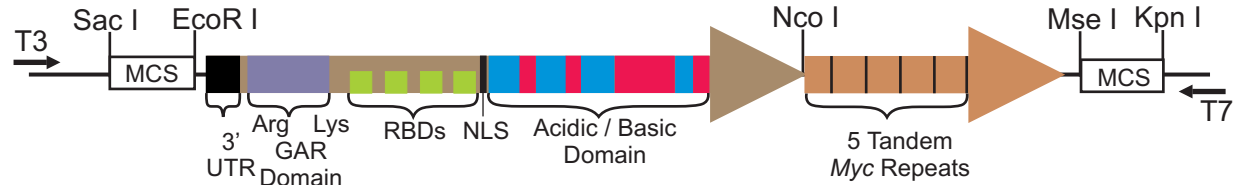
**A Fig. 1.** Vector maps of wild type and Arg→Lys substituted GAR domains from hamster nucleolin. The circular map represents the prokaryotic expression vector pET30a (5422 bp) containing subclones of the GAR domain. The enlarged area is the multiple cloning sequence (MCS) containing either the wild type or Arg→Lys substituted GAR domains from nucleolin.



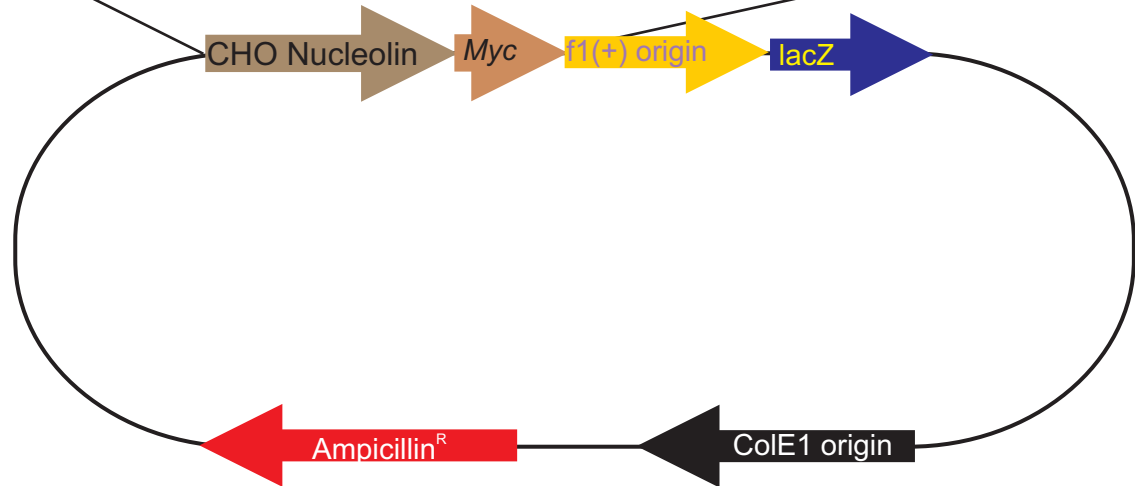
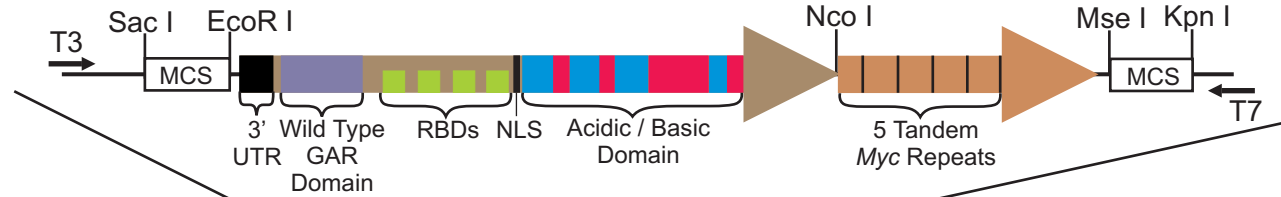
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(5097 bp)



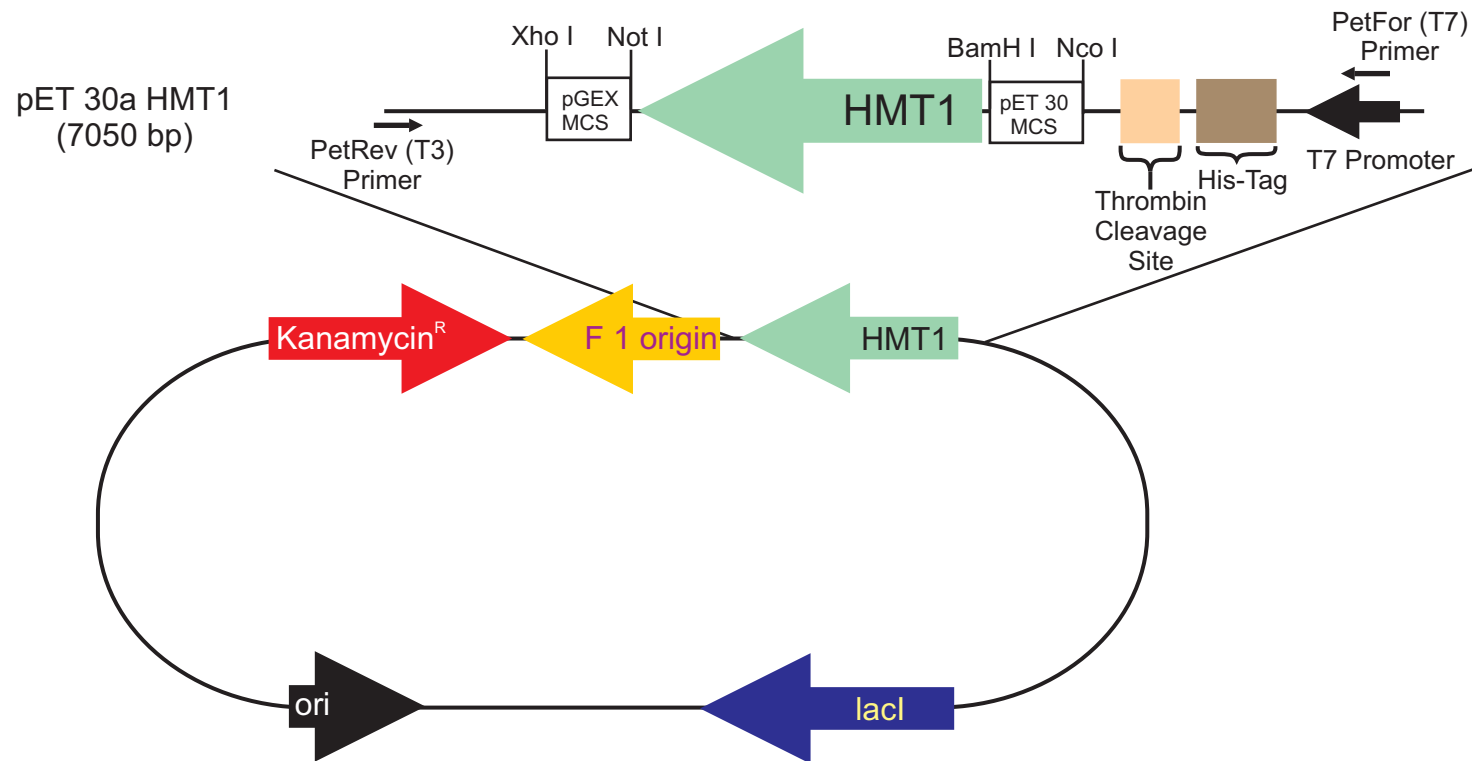
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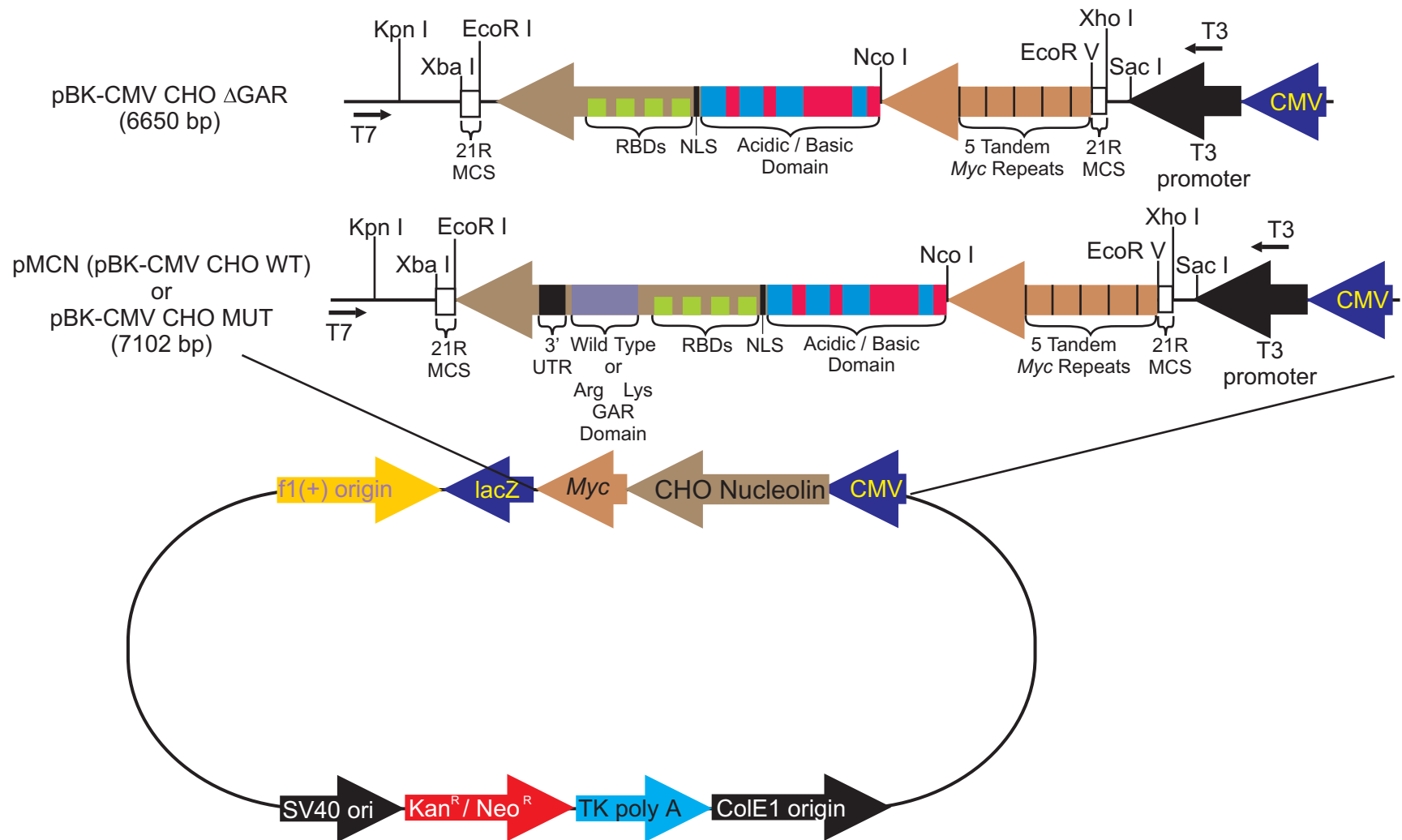
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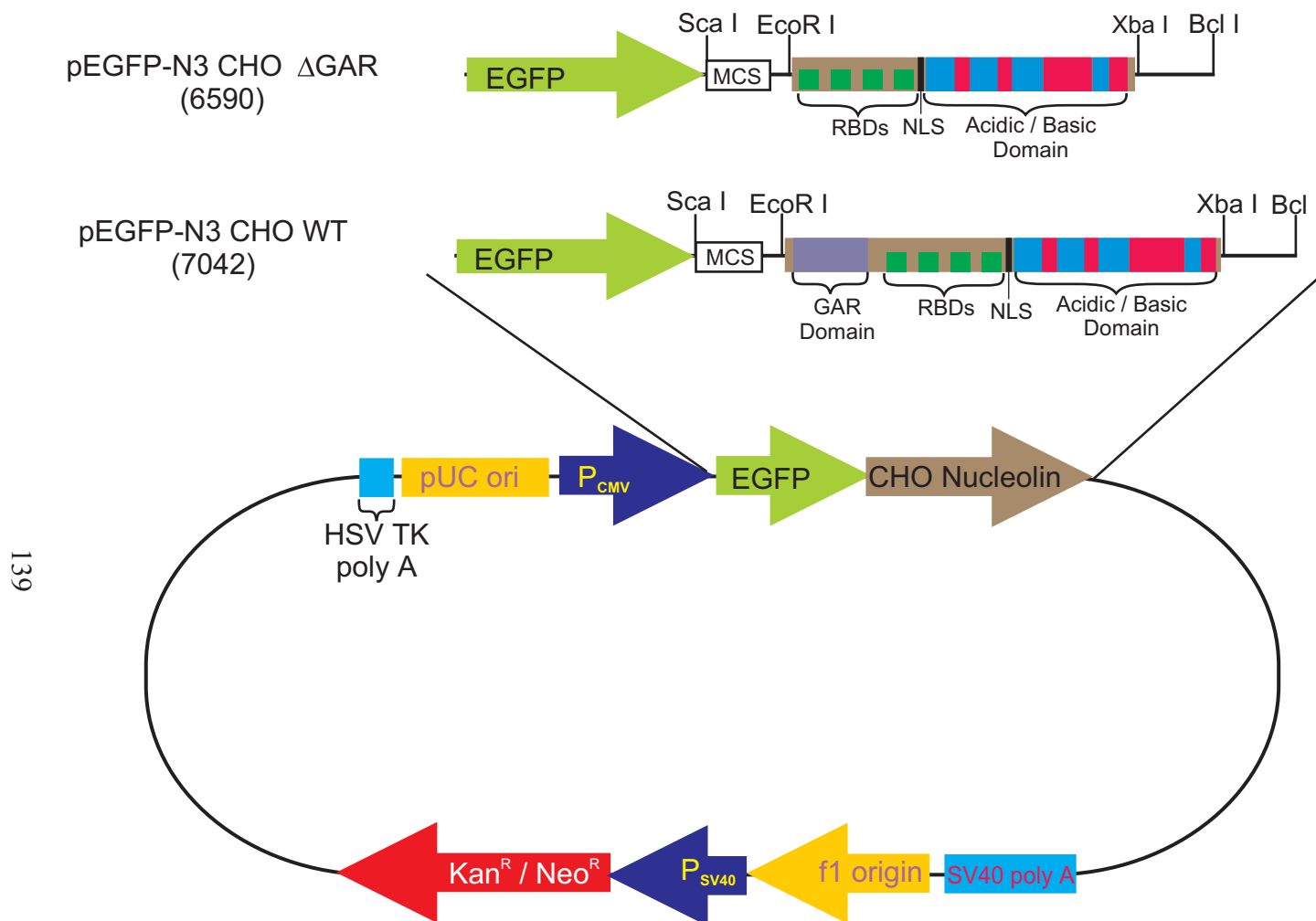
**B Fig. 2.** Vector maps of 21R constructs (hamster nucleolin in *myc*-6-D). The circular map represents pBluescript SK<sup>+</sup> (2961 bp) with one of the hamster nucleolin subclones. The enlarged area is the multiple cloning sequence (MCS) containing either wild type, Arg→Lys substituted mutant, or  $\Delta$ GAR nucleolin with 5 *myc*-repeats at the amino terminus.



**A Fig 3.** Vector maps of *HMT1* in pET 30a. The circular map represents the prokaryotic expression vector pET30a (5422 bp) containing *HMT1*. The enlarged area is the multiple cloning sequence (MCS) containing *HMT1* in frame with the histidine tag.

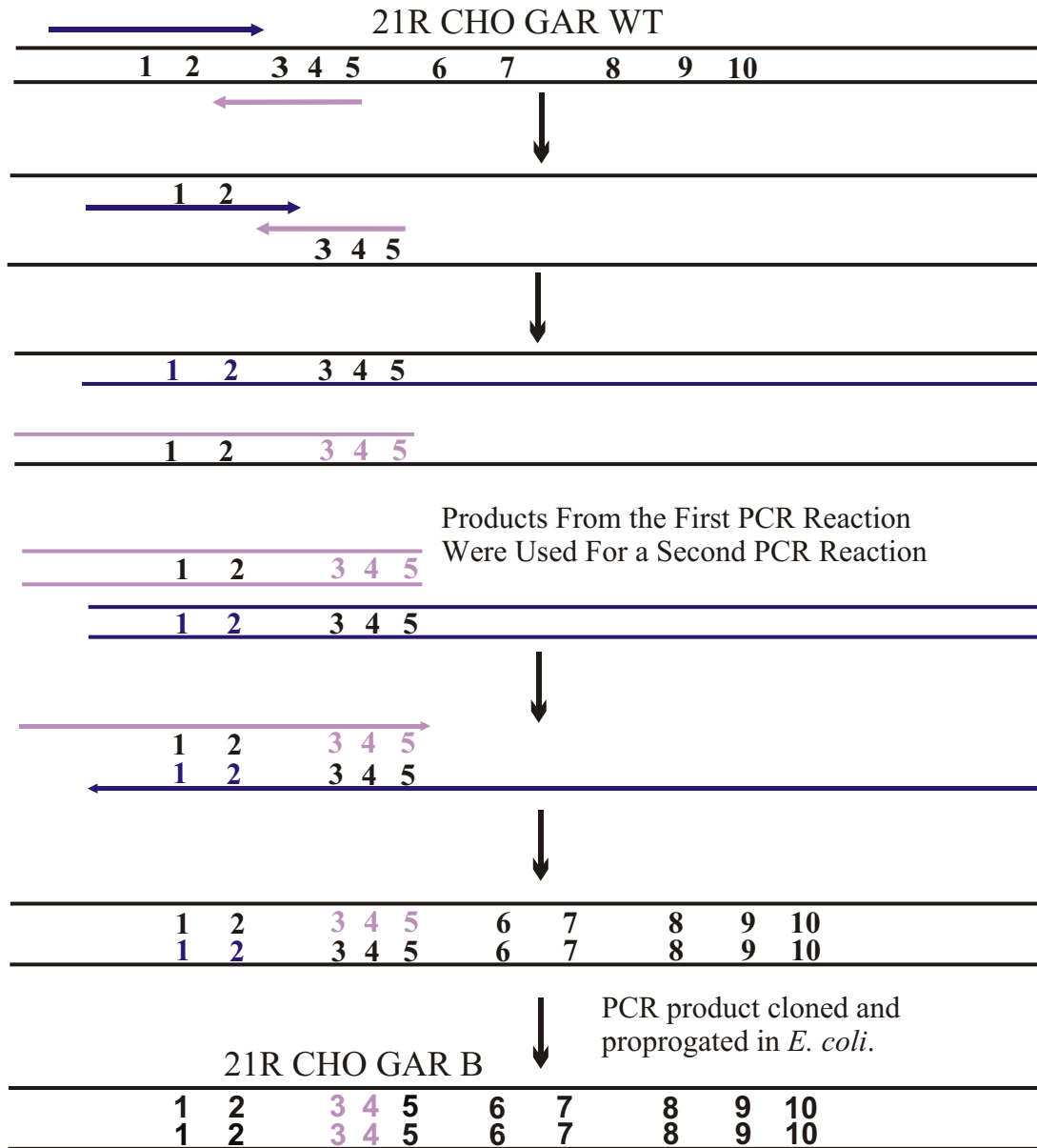


**A Fig 4.** Vector maps of pBK-CMV hamster nucleolin constructs. The circular map represents pBK-CMV (4512 bp) with various *myc*-tagged nucleolin constructs. The enlarged area is the multiple cloning sequence (MCS) containing either wild type or Arg→Lys substituted nucleolin or  $\Delta$ GAR nucleolin with an amino terminal *myc*-tag.

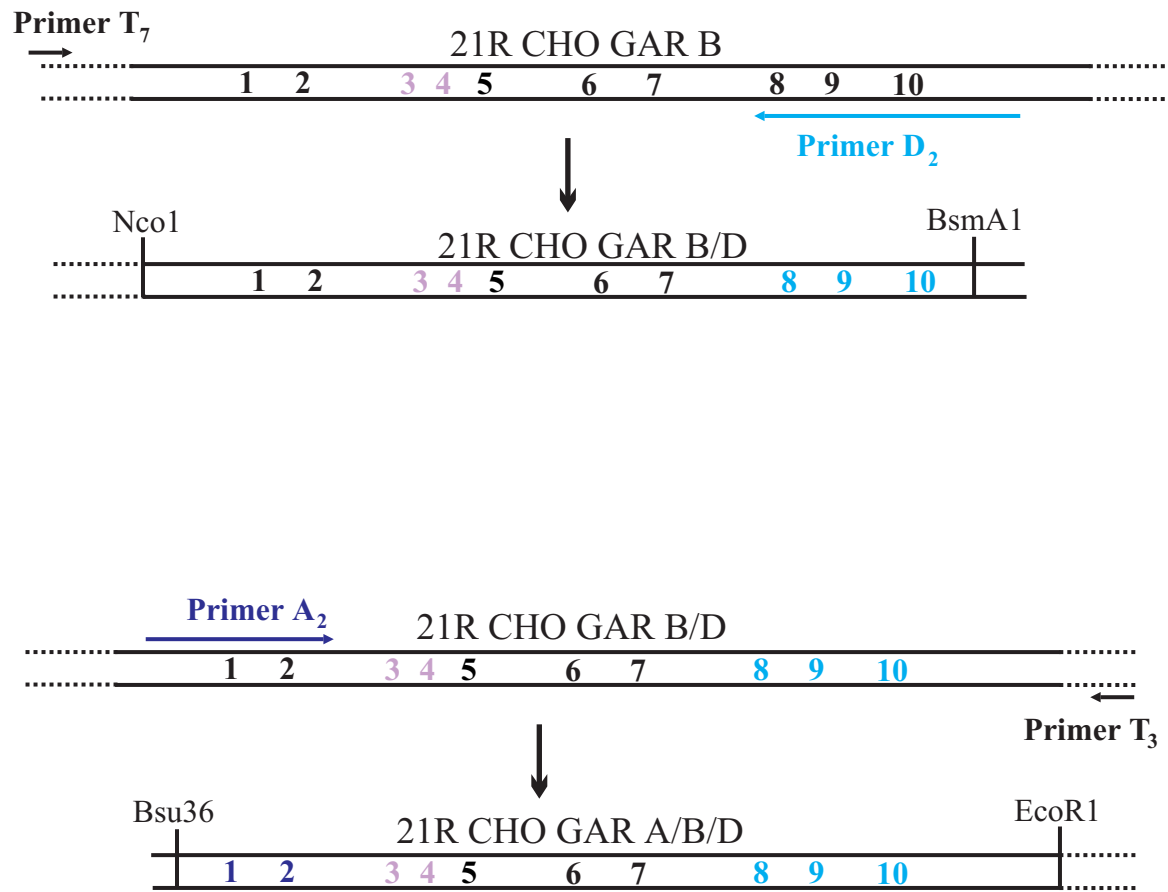


**A Fig. 5** Vector maps of pEGFP-N3 ( $\Delta$ GAR and WT) used to generate stably transfected CHO cells lines. The circular map represents pEGFP-N3 (4700 bp) with either wild type or  $\Delta$ GAR hamster nucleolin. The enlarged area is the multiple cloning sequence (MCS) with wild type or  $\Delta$ GAR nucleolin cloned behind and in frame with the EGFP tag.

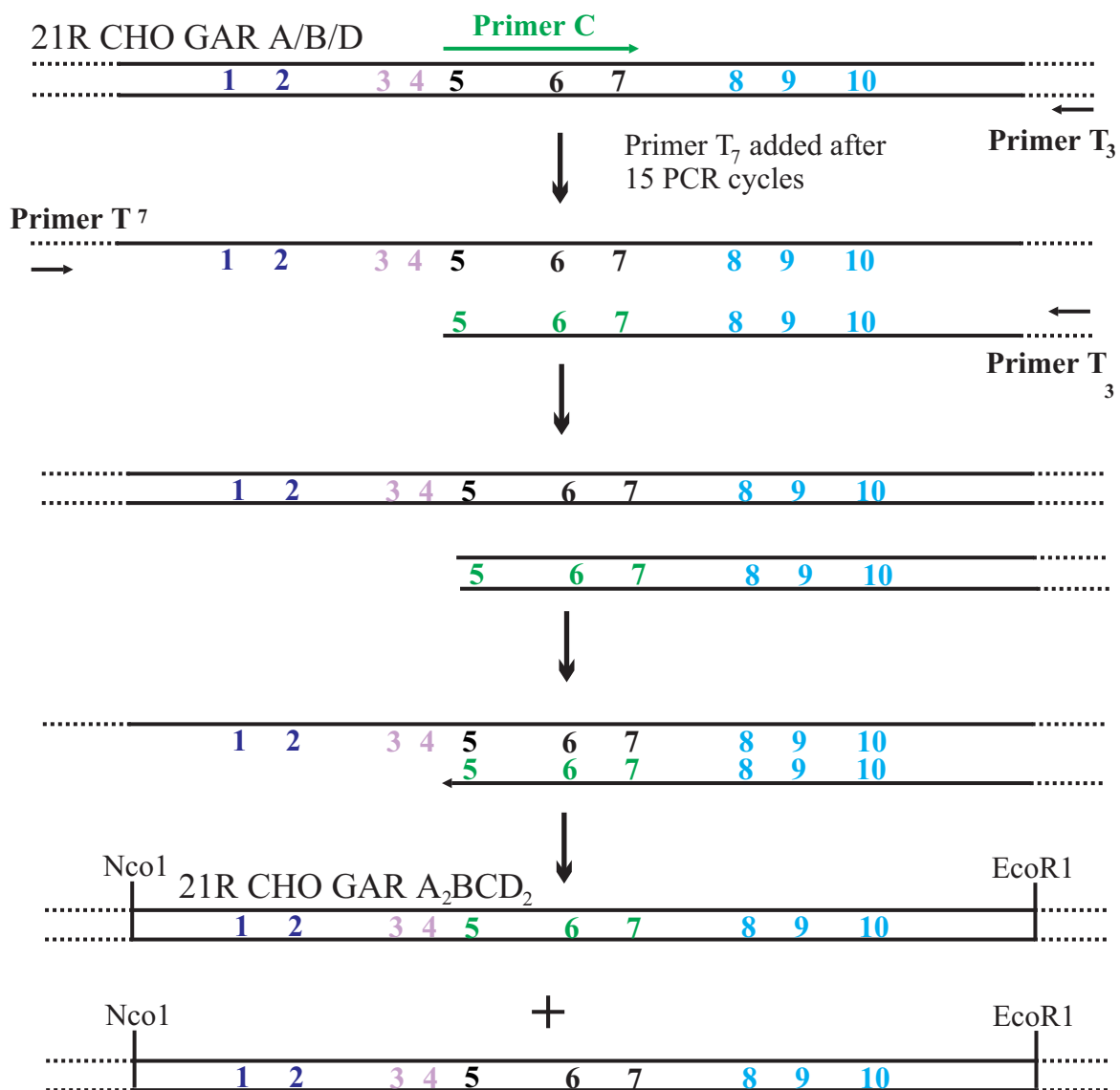
## Appendix B: PCR Strategy



**B Fig 1.** The resulting PCR product was heterogenous DNA containing only one series of mutations in a given strand. The hybrid PCR product was subcloned back into *myc-6-D* using *Nco*I and *Eco*R1 restriction sites. When the resulting plasmid was propagated in *E. coli* the mismatched bases were recognized and repaired by the cell. Each mismatch had a chance of being converted to the matching base of the opposite strand. Sites 1, 2, and 5 were changed to the wild type sequence and sites 3 and 4 were changed to the mutated sequence. This construct was named 21R CHO GAR B.



**Appendix B Fig 2.** PCR strategy used to generate the A and D series of arginine to lysine substitutions. The 21R CHO GAR B/D PCR product was subcloned using NcoI and BsmA1 restriction sites. The CHO GAR A/B/D PCR production was subcloned using Bsu36 and EcoRI restriction sites.



**B Fig 3.** PCR strategy employed for generation of the C series of arginine to lysine substitutions. Primer T7 was added to the PCR reaction after 15 cycles to create a fully substituted product that included a useable restriction site for cloning purposes. The reaction also created DNAs with wild type sequences at the C position. Clones were sequenced to identify a fully substituted clone. The fully substituted clone was named 21R CHO GAR A<sub>2</sub>BCD<sub>2</sub>.

## Appendix C: Consent Form

Greg Pellar  
13587 Honey Drive  
Baton Rouge, LA 70810

March 19, 2002

Dear Mr. Pellar,

I hereby give you, Gregory James Pellar, permission to use figure 1.2 from my dissertation entitled “Two Splice Variants of Nopp140 in *Drosophila melanogaster*” (Copyright registration # TX 5-444-537) for use in your dissertation entitled “The Post-translational Methylation of Arginine in the Glycine Arginine Rich Region of CHO Nucleolin”.

Respectfully,

John M. Waggener, Ph.D.



## **Vita**

Gregory James Pellar attended the University of Mississippi in Oxford, Mississippi, and graduated in the spring of 1994 with a Bachelor of Science degree in biology. After living in Shreveport, Louisiana, for one year, he entered the graduate program in the Department of Biochemistry at Louisiana State University in Baton Rouge, Louisiana, in the fall of 1995. His life in Baton Rouge was highlighted by the birth of his son Geoffrey James Pellar in 2001. Mr. Pellar will graduate with the degree of Doctor of Philosophy in biochemistry from Louisiana State University in May, 2002.